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THE UNIVERSITY OF ALBERTA

ASSAY OF HUMAN PLASMA CATECHOLAMINES

BY

DRAGANA DAMJANOVIC

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

EXPERIMENTAL SURGERY

DEPARTMENT OF SURGERY

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Moja draga Nano*,

Stranice ove teze ilustruju što sam radila u proteklom vremenu umesto da to vreme provedem sa Tobom. Do kraja moga života biće mi žao da ova teza nije bila gotova na vreme i da smo zbog toga, a i nekih "gluposti", toliko malo zajedničkog vremena delile u našem životu. Za utehu kao jedinu misao koju mogu da iskažem je nada da će ovaj rad pomoći ljudima u istoj meri i sa ljubavlju kao što je to ljudima pružao moj pokojni Djedo. Jedan dan bićemo opet zajedno u našem miru.

*(gde god sada bila)

ABSTRACT

Concentrations of catecholamines (CA) in plasma reflect, to a significant extent, the activity level of the sympathetic nervous system but a sensitive means to accurately quantify the CA on a routine basis had not previously been readily available.

The present work has involved the development of a reliable, sensitive and reproducible human plasma CA assay. An efficient boric acid extraction method was used to extract the CA from plasma; the CA were subsequently separated and detected by high pressure liquid chromatography (HPLC) and (amperometric) electrochemical detection (ECD), respectively. An on-line, dedicated integrator was used to obtain and record an objective assessment of the peak heights of the analytes on the chromatogram. The entire assay technique described herein has been optimized to accommodate the operation of the ECD at the high sensitivity level (namely, 1 nanoamp full scale deflection of the ECD response) required by the low levels of endogenous CA found in resting human plasma.

A number of unique features have been incorporated into this assay to achieve and maintain its' sensitivity. These include a novel method of cleaning the HPLC-ECD apparatus on a daily basis with no attendant loss of sensitivity and with a minimal re-equilibration period (i.e., less than one-half hour). Additionally, a novel method of polishing the glassy carbon working electrode (WE) is described. This polishing

technique restores the sensitivity of the WE to its' original status (as determined when the WE is new). Recommendations are made regarding the details essential to the operation of the assay on a regular basis and to the continued preservation of the high level of sensitivity of the assay for routine use.

The performance of this CA assay was tested under the constraints of a clinical setting in an effort to judge the ability of the assay to perform under these conditions and in an attempt to answer a clinical question: are plasma CA concentrations elevated in patients who have suffered a subarachnoid hemorrhage due to rupture of a cerebral artery aneurysm? Based on the data collected in the present instance, no firm conclusions could be drawn about the contribution of plasma CA levels to the development of clinical complications in this group of patients.

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will simply state "Thank-you", and will make the assumption that I have adequately expressed my feelings in person.

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LIST OF ABBREVIATIONS

A = adrenaline [also known as epinephrine, E]

BP = blood pressure

CA = catecholamine(s)

COMT = catechol-O-methyl transferase

CNS = central nervous system

CSF = cerebrospinal fluid

DA = dopamine

DHBA = 3,4-dihydroxybenzylamine

DOE = deoxyepinephrine (or epinine)

ECD = electrochemical detection (or detector)

EDTA = (ethylenediamine)-tetraacetic acid

F.S.D. = full scale deflection

HR = heart rate

HSA = heptanesulfonic acid

HPLC = high pressure liquid chromatography

MP = mobile phase

NA = noradrenaline [also known as norepinephrine, NE]

PNMT = phenylethanolamine-N-methyl transferase

REA = radioenzymatic assay

SP = stationary phase

SAH = subarachnoid hemorrhage

SNS = sympathetic nervous system

$\dot{V}_{O_2}$ = oxygen consumption

WE = working electrode

XRD = X-ray diffraction

INTRODUCTION

A. The Role of Neurotransmitters

One entity responsible for the maintenance of vertebrate life is the nervous system. The vertebrate nervous system can be divided into two main parts: the central nervous system (CNS) which lies within the skull and vertebral column and the peripheral nervous system (PNS) which lies outside the CNS and comprises the twelve pairs of nerves which leave the brain directly. Included in the PNS are the thirty-one pairs of spinal nerves which leave each segment of the spinal cord and intrinsic derivatives of this nervous system such as those of the gut. The CNS communicates with the body of the organism by means of the PNS: the CNS receives information regarding organ function and sensation along afferent neural pathways and it sends instructions out to the body on the efferent side.

Transmission of information along nerves is an electrochemical process: nerves propagate signals electrically via their membranes along their own length. When these signals reach their target (be it another nerve cell or a target tissue) the nerve ending releases chemical messengers ("neurotransmitters") which diffuse to the target and combine with it, thereafter eliciting responses within it. The electrical nerve signal itself is known as an action potential and has a duration of about one millisecond. Each action potential releases a quantum of neurotransmitter and

information is coded by the frequency and pattern of the electrical impulses.

The peripheral nervous system has two large subdivisions: the somatic and autonomic nervous systems. The somatic nervous system comprises largely voluntary motor neurons to skeletal muscles and sensory neurons. The autonomic nervous system (ANS), on the other hand, regulates the activities of structures that are not under voluntary control and that, as a rule, function below the level of consciousness. Therefore, respiration, circulation, digestion, body temperature, metabolism, sweating and secretions of certain endocrine glands are regulated in part, or entirely, by the ANS. Obviously, the integrated activity of the ANS is of vital importance to the well-being of the organism.

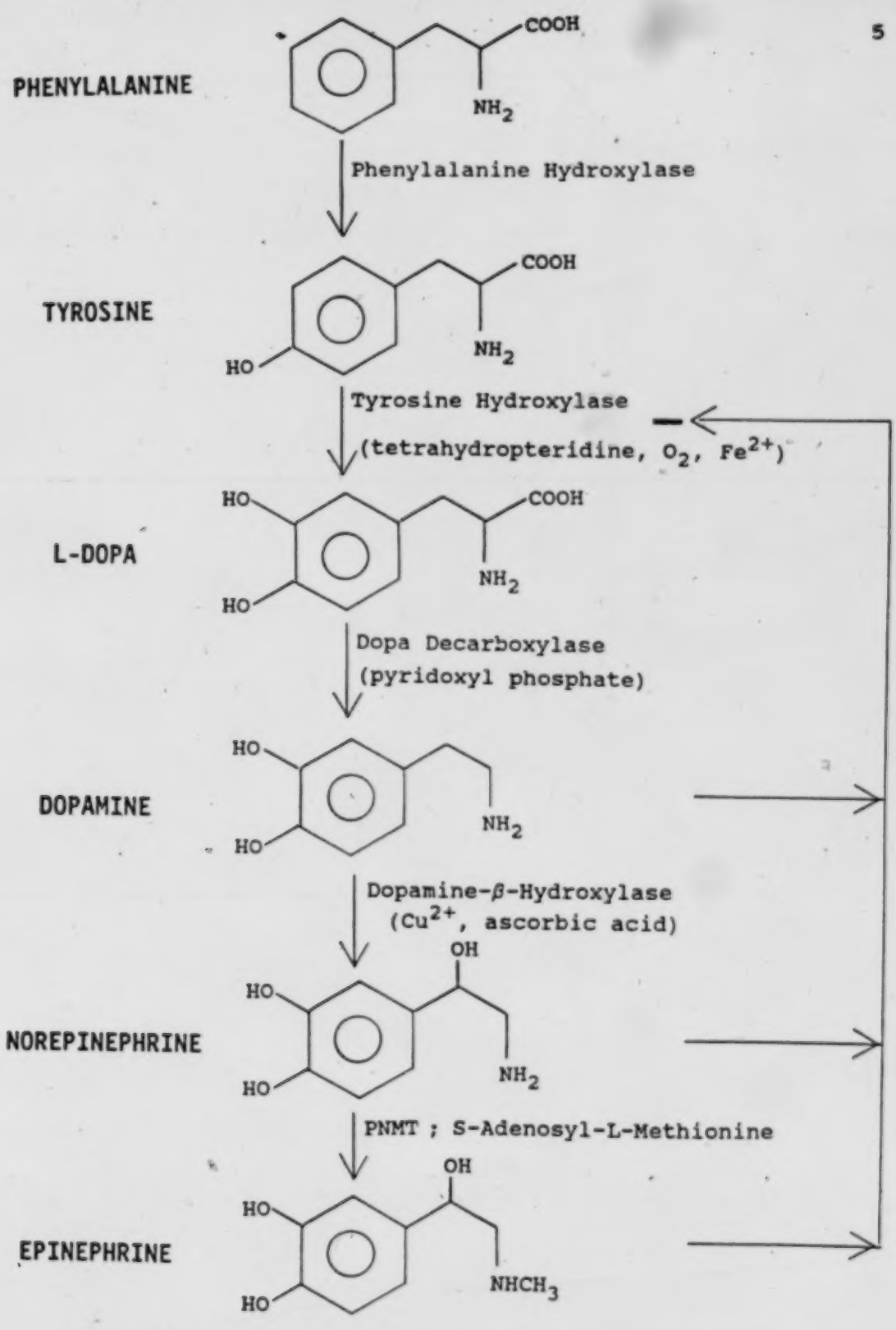
The ANS is a self-regulating system. It functions by means of a balance between two systems which can be viewed as an "accelerator" and a "brake". That is, instructions are sent in an "ON-OFF" code with the end result being an integration of the signals received since most structures are innervated by both of these systems. Generally speaking, "OFF" instructions are sent along parasympathetic nerves (using the neurotransmitter, acetylcholine) and "ON" instructions are sent along sympathetic nerves (using the catecholamines as neurotransmitters). The designation "catecholamine" refers to a compound composed of a catechol nucleus (a benzene ring with two adjacent hydroxyl groups) and an amine-containing side chain. [The parent compound is β-

phenylethylamine.] The most important catecholamines (CA) known to occur in humans are dopamine (DA), noradrenaline (NA) and adrenaline (A) [See Figure 1].

The parasympathetic nervous system is concerned with the functions of conservation and restoration of energy (i.e. vegetative aspects of day-to-day living) and is organized for discrete and localized action rather than for mass response. It will slow down the heart rate (hence the "brake" analogy), lower the blood pressure, stimulate gastrointestinal movements and secretions, aid absorption of nutrients, protect the retina from excess light and empty the urinary bladder and rectum.

The sympathetic side of the ANS is concerned with the expenditure of energy and the coordination of processes which allow the organism to deal with stress (hence, the "accelerator" analogy). The principal neurotransmitter of this system is noradrenaline [NA] (or norepinephrine [NE], as it is also called) and the network is often referred to as the adrenergic system. Among other effects, the sympathetic nervous system (SNS) accelerates the heart, raises the blood pressure, dilates the bronchioles, inhibits peristalsis of the intestines, causes the breakdown of glycogen into glucose and the liberation of free fatty acids (thereby supplying energy), and shifts blood flow to skeletal muscle. These actions may be significantly amplified by the liberation of adrenaline [A] (or epinephrine [E], as it is also called) from the adrenal medulla into the bloodstream. [The adrenal medulla may be

Figure 1. Metabolic cascade for the synthesis of catecholamines.
PNMT = phenylethanolamine-N-methyltransferase.


PHENYLALANINE
COOH
NH2
Phenylalanine Hydroxylase
TYROSINE
COOH
HO
NH2
Tyrosine Hydroxylase
(tetrahydropteridine, O2, Fe2+)
L-DOPA
HO
COOH
HO
NH2
Dopa Decarboxylase
(pyridoxyl phosphate)
DOPAMINE
HO
HO
NH2
Dopamine-β-Hydroxylase
(Cu2+, ascorbic acid)
NOREPINEPHRINE
OH
HO
HO
NH2
PNMT ; S-Adenosyl-L-Methionine
EPINEPHRINE
OH
HO
HO
NHCH3

regarded as a specialized sympathetic ganglion, adapted for the release of A directly into the bloodstream.] The sympathoadrenal system, then, is organized in such a way so that the structures which it innervates can be orchestrated simultaneously and with great force. It is to be appreciated that, in mammals, an action potential can travel as fast as 120 m/sec along a nerve fiber ("axon") whose diameter is, in turn, measured in Angstroms (Å, 10^{-4} μm). [Axons are no larger than 20 μm in diameter and originate from a nerve cell body which, in turn, is about 30 μm wide (mammals).] The sympathoadrenal system functions continually but the degree of activity varies from organ to organ and from moment to moment. Thus, fine adjustments can be made in a constantly changing environment. [For a more detailed discussion of these fundamental neurophysiological principles, the reader is referred to Kuffler et al., 1984.]

It will be obvious from the preceding discussion that NA and A are intimately involved in the day-to-day maintenance of normal vertebrate life. These two CA are also thought to play a role in many pathological processes which disrupt the function of the structures and/or organs modulated by them. In the CNS, DA functions as a neurotransmitter but is of interest in the periphery mainly as a precursor of NA. Therefore, quantification of both NA and A would, at least potentially, provide an intimate window to the state of an organism from a sympathetic nervous system point of view. As well, periodic monitoring of NA and A would yield a dynamic

assessment of that state. Thus, the importance of quantification of NA and A as a clinical and research tool cannot be overstated.

From an experimental point of view, there are a number of possible approaches to accomplishing the task of quantifying NA and A. The neurotransmitter outflow of sympathetic nerves spills over into the various physiological fluids (including blood plasma, cerebrospinal fluid and urine). [Hoeldtke, et al., 1983; Ziegler, et al., 1977]. Assaying of these fluids for their CA content can provide information generally not available through other techniques. For example, while it is technically possible, to a limited extent, to study the sympathetic nerves to skin and skeletal muscle by microneurographic electrophysiological methods, the nerves to internal organs are not accessible for such testing. However, the variations in sympathetic neurotransmitter activity of internal organs can be assessed by biochemical means, namely by the measurement of the CA content of physiological fluids. By so doing, it should be possible to at least infer the level of nerve activity in the organ(s) of interest and under the conditions of study [Esler et al., 1985].

The following summary of the life cycle of the CA is given in the interest of providing background to the significance and interpretation of the research projects described thereafter.

B. Synthesis and Secretion of Catecholamines (CA)

CA are produced and released into physiological fluids through the following mechanism. From an anatomic point of view, nerves lie in very close proximity to blood vessels throughout the body. The amino acid, tyrosine, leaves the blood and enters the nerve terminal varicosity by a special concentrating mechanism. Tyrosine is then converted in the cytoplasm to L-Dopa by the enzyme tyrosine hydroxylase (Figure 1) (which is found only in CA-producing cells). This reaction proceeds very slowly in vivo and is considered to be the rate-limiting step in the biosynthesis of the CA. Tyrosine hydroxylase is inhibited by CA and this feedback inhibition appears to be important in controlling the rate of biosynthesis of NA in the sympathetic nerves. L-Dopa is, in turn, rapidly decarboxylated to DA by the enzyme Dopa decarboxylase. The DA then enters minute, granulated vesicles (400-600 Å size) in the sympathetic nerve terminals where it is finally hydroxylated by dopamine-β-hydroxylase (DβH) to L-NA. DβH is found only in cells that produce NA. [In the adrenal medulla and in some neurones in the CNS, NA is further converted to A as a result of methylation of the amine group by PNMT (or, phenylethanolamine-N-methyl transferase).] The NA and A remain protected and inactive inside the storage vesicles until liberated by an action potential. [For a more detailed discussion of CA metabolism the reader is referred to Bradford, 1986.]

Action potentials are caused by the penetration of Na^{+} together with Ca^{++} into the neuroplasm. As a consequence of the increased intraneuronal Ca^{++} concentration, the CA-containing vesicles migrate toward and fuse with the neuronal cell membrane and expel their content of NA into the synaptic cleft. The neurotransmitter diffuses across the gap to combine with specific receptors on the post-synaptic membrane and thereby elicits responses in the post-synaptic target (or effector) cell. [For a more detailed discussion of synaptic function, the reader is referred to Edelman et al., 1987.]

C. Inactivation of the CA

Inactivation of the CA can occur by several different pathways. The primary one is re-uptake of the CA back into the same nerve terminal varicosity that only moments before had released them. Once back inside the terminal, the CA molecules are re-stored in the vesicles and thus recycled. This is a highly specific, high-affinity and very rapid process called "Uptake 1" (or, Neuronal Uptake). Under normal conditions, this pathway predominates. However, when very high levels of CA are released from the neurons by, for example, continuing stimulation of the adrenal medulla (or by intravenous injection of CA), then a significant proportion of the released CA will be removed by re-uptake into non-neuronal tissues such as muscle, connective tissue, liver and kidney. This is called "Uptake 2" (or, Extra-Neuronal Uptake) and in this case, the molecules are catabolized by enzymes. The two principal enzymes which digest the CA are monoamine oxidase

(MAO) and catechol-O-methyltransferase (COMT). [For a more detailed discussion of CA catabolism and inactivation, the reader is referred to Cooper et al., 1986.]

Primarily because of the efficiency of these uptake mechanisms, only a small fraction (perhaps 10-20%) of the physiologically active CA released by the nerve terminals reaches the receptors on the target cells and activates them [Bevan et al., 1980].

D. Significance of Plasma Studies of CA

As was indicated earlier, nerves lie in close proximity to blood vessels; therefore, neurotransmitter spillover that escapes uptake or degradation will rapidly appear in the blood stream [Whitby et al., 1961; Yamaguchi et al., 1977]. The same is true of extracelluar and cerebrospinal fluids. In accordance with the preceding discussion on the metabolism of CA, one would also expect to find CA metabolites in these fluids and this is, indeed, the case.

Urine, on the other hand, is essentially an ultrafiltrate of plasma and is collected over a period of time, up to several hours. Compounds in urine depict activities taking place throughout the body, including the brain. For this reason, CA metabolites (as opposed to the parent molecules) predominate in the urine and thus represent an integrated image of many events which have taken place in the past [Kopin, 1985]. Furthermore, CA metabolites in urine have time to accumulate to the nanogram per mL concentration range.

Metabolites are usually more stable than the parent molecule and this is especially true in the case of CA; this fact has important implications for CA assay techniques.

The choice of physiological fluid to sample for CA spillover is based on the kind of information about the body that is required. Most often, a plasma assessment is preferred because of the dynamic nature of the SNS and the virtually immediate reflection of this by CA spillover into plasma. In both clinical and research settings, one is trying to gain information about a system that is highly influenced by the state of the organism at that point in time and a particular state of health. However, to provide meaningful data from assays of plasma CA, it is necessary to balance the dichotomy of physiological dynamicism and methodological limitations.

Until recently, there have been severe limitations on the ability to quantify the concentration of plasma catecholamines, in part due to the ethereal quality of the CA themselves. That is, the CA are small compounds (less than 200 molecular weight) and are inherently unstable, having a half-life in plasma of only about two minutes [Esler et al., 1979]. Furthermore, CA are present in extremely low concentrations, viz., picograms per mL of plasma [150-300 pg/mL for NA and 20-80 pg/mL for A].

It is important to realize that only a small proportion (perhaps 20%) of the NA released by the action potential escapes by diffusion into the bloodstream (and other

physiological fluids). Once there, some of the NA is converted to sulfates and glucuronides (about 80%, in fact) and the remainder is left free (about 20%). It has been demonstrated that it is the free CA fraction which fluctuates in response to stimuli; therefore, measurement of this fraction constitutes a valid appraisal of CA and ANS dynamics [Bravo & Tarazi, 1982]. The exact function of the sulfoconjugated CA is not known and the mechanisms of the equilibria which create and maintain this pool remain to be elucidated [Kuchel et al., 1985].

The amount of neurotransmitter that actually escapes from the synaptic cleft will depend, at least in part, upon the sympathetic nerve firing rate, the density of the sympathetic nerves in that organ or area, the width of the synaptic cleft, the capacity of neuronal and non-neuronal tissues for re-uptake and/or enzymatic degradation, the permeability of the local capillaries to the CA, and the blood flow through the organ [Esler et al., 1985]. Obviously, pharmacological agents can act at a number of sites along this cascade of events thereby altering these proportions once again. Nonetheless, it is the fact that CA are released by nerve cell activity and subsequently appear in the body fluids that is the basis for the attempt to use measurements of CA concentrations in samples of body fluids as an "index" of SNS activity.

It should also be apparent that, for example, plasma levels of NA and A can and will fluctuate rapidly in response to a variety of stimuli which provoke a sympathetic response

[e.g., Watson et al., 1979]. This fact alone places constraints on the design of experiments investigating the functioning of the SNS. Some of the limitations imposed thereby are: first, the assessment (in whatever form it might assume) cannot disrupt normal function or in any way alter it (i.e., affect the sympathoadrenal system); secondly, by implication, the technique must be as non-invasive as possible and be repeatable and reproducible; thirdly, from a practical point of view, the assessment should be realistic in terms of having a minimum of special equipment and/or handling requirements.

In spite of the above-mentioned complications and limitations, there is general consensus that peripheral venous plasma determinations of CA concentrations can provide useful clinical and research-related information as long as studies are properly controlled [Hjemdahl, 1986].

E. CA in Plasma

The preceding review has established the rationale and importance of being able to quantify plasma CA. However, from where should one obtain this plasma (blood) sample? Logically speaking, the sample should be from the venous outflow of the organ being studied. Yet one is then forced to make the assumption that no NA came into the organ (on the inlet, or arterial side) and that the organ did not extract any NA that may have been present there. These may or may not be safe assumptions. Furthermore, selective catheterization of

individual organs is not normally practical. What is practical is a venous blood sample from a peripheral vein. However, if many organs are spilling excess CA into the blood from a wide range of physiological tasks, it would seem that such a blood sample would not provide any useful information. Fortunately, it does. The venous blood levels of NA and A do parallel the (verifiable) level of sympathetic activity in a number of real-life and experimental situations [e.g., Wing et al., 1977]. Even though what is being sampled is some net result of events occurring throughout the body (including the brain), the blood levels of NA and A do fluctuate in a coherent fashion.

For example, at rest, the normal plasma level of NA is about 150-300 pg/mL and A is about 25-75 pg/mL. However, during exercise, these values (short-lived though they are) can climb to 5,000 pg/mL for NA and 1,500 pg/mL for A [Lehmann et al., 1981]. Occasionally, a patient with a tumour of the adrenal medulla (known as a pheochromocytoma) will have plasma levels of NA at 10,000 pg/mL and A at 4,000 pg/mL [Bravo et al., 1981]. This latter amount of A is essentially lethal, as is the tumour if allowed to grow unchecked [Bravo & Gifford, 1984]. Since these changes in plasma concentrations of CA are at the level of orders of magnitude, it is safe to conclude that by measuring plasma levels of CA, we have a useful "index" of sympathetic nervous system activity.

It is important to remember that A is mainly synthesized as a hormone in the adrenal medulla. In that case, any

increase in the concentration of A in plasma will indicate increased adreno-medullary secretion. NA, on the other hand, is mainly a neurotransmitter released by post-ganglionic sympathetic nerve endings. At low impulse rates, about 90% of the NA released by the nerve terminal is removed by re-uptake back into the same nerve terminal. It is only at high nerve impulse rates (or breakdown of the re-uptake system) that higher amounts of NA can escape into the bloodstream. It follows that this is the explanation for the low, resting levels of plasma NA and A that are normally encountered and that plasma CA levels will be a more reliable index, or indicator, of total adrenergic activity the more intense the adrenergic stimulus [Koch et al., 1982].

It is obvious from the above that if one wishes, by monitoring fluctuations in plasma levels of NA and A, to study the participation of NA and A in maintaining homeostasis in an intact organism, there are some major limitations. The experimental design must take into consideration that the sympathetic response to stimuli is organ-, and tissue-specific and is highly variable within individuals and between individuals. Furthermore, the physical and psychological state of the individual (including stress and body-positioning) and recent activities undertaken must be known. The sampling site of the body fluid to be examined is a critical issue. The age of the individual is also important because, for example, it is known that while the concentration of NA in plasma increases with increasing age, this is due to

diminished clearance rather than increased production. From an experimental point of view, therefore, it is critical that an individual act as his/her own control and that he/she is followed for a period of time under standardized control and experimental conditions [Hjemdahl, 1986].

Under special circumstances such as in a catheterization laboratory, it is possible to study regional sympathetic response patterns. Note that measurement of plasma levels of CA is always accompanied by the simultaneous collection of other physiological data such as heart rate, blood pressure, oxygen consumption and the like. Taken in isolation, CA concentrations are usually relatively meaningless although they may have some meaning if, as suggested above, individual subjects act as their own controls (because CA responses are so individually tailored to the needs of a single organism). Otherwise, pooling of such individual, raw CA data renders potential results meaningless [Holly and Makin, 1983].

F. The Nature of CA Studies

It will be obvious from the preceding discussion that a number of important parameters must be established prior to the utilization of plasma CA levels as indicators of underlying physiological mechanisms. These include the establishment of the reproducibility of plasma CA levels under standardized conditions of physiological status (CA concentrations may not fluctuate reproducibly when compared with other physiological markers [Cronan & Howley, 1974]), the

characterization of the CA response to stressors (e.g., mental arithmetic or an exercise stimulus), the differentiation of a normal from an abnormal CA response to a physiological challenge and acute versus chronic alterations in CA dynamics, and all of the implications thereof. Additionally, seasonal (and circadian) variations in plasma CA responsiveness and dynamics must be known and controlled for appropriately. All of these factors have serious implications for studies (both clinical and research-oriented) aimed at elucidating sympathetic control mechanisms in intact humans. And, before any studies of SNS function, as reflected by CA concentrations, can be attempted, the above information must be established with certainty.

One of the most critical issues is the reproducibility of the plasma CA response to a stimulus. While this issue has been raised on numerous occasions in the literature, it is not normally an element of the design of individual studies and it is rarely established in the experimental population [e.g., Lehmann et al., 1981]. The assumption has traditionally been made that the magnitude of the plasma CA responses to stimuli is an identical and invariant phenomenon. However, there have been indications in the literature that this is not the case [see Appendix I; Ward et al., 1983; Hyyppä et al., 1986]; yet this fact has received little attention. The ramifications of this concept, however, are pertinent to any study of plasma CA concentrations: if the plasma CA response to a stimulus is not reproducible, then the value or usefulness of plasma CA data

may be jeopardized even if the other physiological variables have been controlled or standardized as described above.

Unfortunately, a major limitation to the study of such adrenergic physiology and pathophysiology *in vivo* has been methodologic: we have been unable to quantify plasma CA accurately. The exact reasons for this state of affairs will become apparent in the next section.

G. Measurement of CA Concentrations

The rest of this introductory section will be concerned with the measurement of the levels of CA found in human plasma samples. This model (i.e., human plasma CA) is used because this particular analysis presents the greatest analytical challenge to the development of practical and useful CA assays. Each one of the CA has different physiological and physico-chemical properties and this latter characteristic has been exploited to develop assay techniques (below). Historically, these assays have proven to be a challenge both from the point of view of the compounds being tested and from the point of view of the technology available.

The CA are small molecules (less than 200 molecular weight) with very subtle structural differences [see Figure 1]. CA are normally present in very low quantities in plasma (cf., picograms of the native CA per mL of plasma versus nanograms of stable metabolites in similar volumes of urine or nanograms of CA per gram of certain tissues). CA are unstable molecules which decompose in alkaline solutions, upon exposure

to light and even if brought to temperatures greater than +5°C. Therefore, before plasma CA concentrations can be quantified, the CA must first be stabilized, separated from the other constituents of plasma and from each other.

A variety of assay techniques, and combinations of techniques, have been tried to achieve the accurate quantification of CA. Very few of these methods have proven to be practical, and progress in the field of SNS research has been limited by these technical difficulties. [For a more detailed discussion of CA assay methodologies, the reader is referred to Baker and Coutts, 1982.]

Since the 1950's, there have been three major revolutions in available technology and all have resulted in improved CA assays. These methods represent attempts to improve the specificity and sensitivity of CA measurements while reducing interference from other compounds. They are:

(1) Development of the fluorometric assay (mid-1950's to mid-1960's);

(2) Development of the radioenzymatic assay (mid-1960's to mid-1970's);

(3) Development of an assay utilizing high pressure liquid chromatography with electrochemical detection (HPLC-ECD) (late 1970's to present) [see Appendices II & III].

Other methods of separation and detection of CA have been tried including Gas Chromatography-Mass Spectrometry (GCMS), radioimmunoassays [Raum, 1987] and a variety of techniques employing detection of the CA by electron-capture detectors, ultraviolet absorption and fluorescent spectroscopy (with and without liquid chromatography) but none of these has, to date,

proven of practical value [Holly and Makin, 1983; Krstulovic, 1982].

Of the three principal methods, the HPLC-ECD assay is the best option from the point of view of reliability, reproducibility, efficiency and sensitivity [Krstulovic, 1982]. To put this method into perspective, a short review of all three methods will be presented.

(1) Fluorometric Assay

The fluorometric assay is based on the fact that CA molecules possess natural fluorescence [Krstulovic & Powell, 1979]. The CA and their metabolites have characteristic emission spectra which can be exploited, to some extent, to differentiate between them. The idea behind an assay based on this fact was to be able to detect levels of CA in physiological samples with a minimum of pre-treatment (i.e., only isolation from plasma). [Normally, the plasma was only de-proteinized prior to analysis.] However, not only is the native fluorescence of the CA very weak and non-specific, but these molecules are present in such low concentrations in plasma that they can barely be detected by available instruments. This limitation can be overcome by the derivatization of the CA to larger molecules which have strong native fluorescence (e.g., trihydroxyindoles and o-phthalaldehyde derivatives) thereby rendering the CA more visible and facilitating their detection.

One of the major advantages of this method is that it is quick to perform and comprises relatively few steps;

additionally, the equipment required is inexpensive and readily available. Among the major disadvantages of this type of assay is the complication that plasma contains many compounds (other than those of interest) and some of these are susceptible to the isolation and concentration procedures employed. Such compounds may also fluoresce, thereby invalidating the assay. Compounds embodied within many common foods, such as bananas, chocolate and coffee, fall into this category and interfere with the assay.

Furthermore, even with derivatization, the concentrations of CA in resting plasma (i.e., plasma collected from resting, unstimulated subjects) are so low as to be undetectable. An additional problem with the derivatization is that the molecules thus created are very unstable and must be synthesized and quantified under strictly controlled conditions or else they will, literally, disappear. It is also not possible to differentiate between the different CA and, therefore, the assay can only quantify the total amount of CA present rather than characterize the individual concentrations of NA, A and DA. In time, the assistance of alumina extraction, isotope-labelling and chromatographic separation were added to the fluorometric procedure but neither the specificity nor the sensitivity of this assay method could be significantly improved without heroic efforts. The recent introduction of laser technology to the field of fluorescence detection is expected to improve the sensitivity of this assay method. However, the problem of the stability

of the analytes, and the specificity, sensitivity and reproducibility of the fluorometric assay must still be overcome.

All of these restrictions have precluded the utility of this method for the measurement of plasma CA levels. Therefore, this method is largely reserved for the analysis of urine samples which contain nanogram quantities of CA metabolites [Randolph & Stabler, 1986].

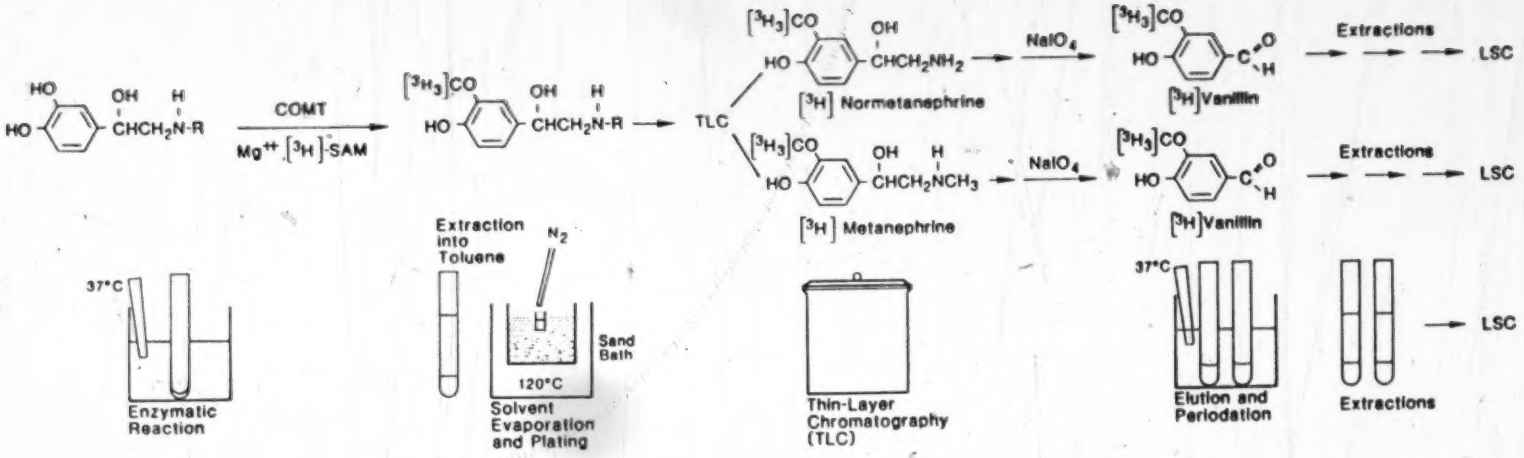
Clearly, the fluorometric assay does not constitute a resolution of the problems of effectively extracting, separating and detecting the individual CA of human plasma. The next major attempt at solving these problems entailed the enzymatic conversion of the CA to their more stable metabolites within a radioenzymatic assay [Newsholme & Taylor, 1968].

(2) Radioenzymatic Assay (REA)

In this method, the CA are converted to their more stable metabolites by the action of their natural enzyme, catechol-O-methyltransferase (COMT) [Newsholme and Taylor, 1968]. The CA metabolites are rendered "visible" by the use of [^{3}H]-S-Adenosyl-L-Methionine, or [^{3}H]-SAM, as the (radioactive) methyl group donor which is supplied to the enzymatic reaction. The radio-labelled metabolites are then extracted from plasma using organic solvents and separated by thin-layer chromatography (TLC) [Figure 2].

[Both the radioenzymatic and HPLC-ECD (below) CA assays utilize chromatography. Briefly, chromatography is a method

Figure 2. Schematic diagram summarizing the radioenzymatic catecholamine assay technique.



COMT: Catechol-O-Methyl-Transferase

[3H]-SAM: [3H]-S-Adenosyl-Methionine

3-4 Hours	4-5 Hours	3-5 Hours	4-5 Hours	4 Hours
Day 1	Day 1	Day 1	Day 2	
* Full Assay: 16 tubes	* Full Assay: 16 tubes	* Full Assay: 16 tubes	* Full Assay: 32 tubes	

by which the constituents of a mixture can be separated away from each other. Once separated, these constituents can then be quantified. The reader is referred to Appendix III for a more complete explanation of the underlying principles of chromatography.]

Each band on a thin-layer plate contains one of the resolved CA, now stabilized and radioactively "tagged"; these bands can be scraped off and subjected to further reactions, as necessary. If desired, the DA can be directly quantified from the radioactivity found in its band by putting the band (silica and all) into liquid scintillation counting fluid and counting the emulsion. [^{3}H]-Normetanephrine and [^{3}H]-Metanephrine are normally further purified by conversion to [^{3}H]-Vanillin by reaction with periodate. The radioactivity of the vanillin is measured and calculations are performed to give the concentration of the original compounds in the plasma [Peuler and Johnson, 1973; Hörtnagl, Benedict et al, 1977].

The major advantages of the radioenzymatic method are that it requires less than one mL of plasma to perform the assay (in duplicate), and that the low resting levels of CA in human plasma can be detected and quantified with accuracy.

The disadvantages of the radioenzymatic method are numerous. For one thing, until recently, the COMT used in the first part of the assay had to be isolated in the researcher's own laboratory. The isolation technique is lengthy and laborious with no guarantee of success [Mason and Weinkove, 1984; Coward et al., 1973; Nikodijevic, 1969 and 1970]. The

isolation is also unpredictable, giving limited control over yield and activity of the enzyme. Secondly, the REA itself has many complicated steps, requires many pieces of equipment and may take as long as two and a half days to complete (See Figure 2) [Hörtnagl et al., 1977]. Lastly, the procedure demands a high degree of technical skill on the part of the operator. Predictably, the assay is also expensive to maintain.

Even if all of the above factors could be controlled, the radioenzymatic assay has two weak points that lie outside of the control of the operator: the quality of the [^{3}H]-SAM is critical to the success of the assay [Mason and Weinkove, 1984; Oldham, 1970] as are the TLC plates [unpublished observations]. Unfortunately, these are both commercial items and thus subject to the vagaries of commercial quality control. Over a longer period of time, it often happens that things go wrong at the manufacturing level (particularly with the [^{3}H]-SAM, a very unstable product [Oldham, 1970]) and a CA assay laboratory can be shut down for months with no solution in sight. This has proven unacceptable to individuals and institutions which might otherwise use the assay on a regular basis. Therefore, the REA does not present a practical solution to the problem of the routine measurement of plasma CA levels.

(3) High Pressure Liquid Chromatography with Electrochemical Detection (HPLC-ECD) CA Assays

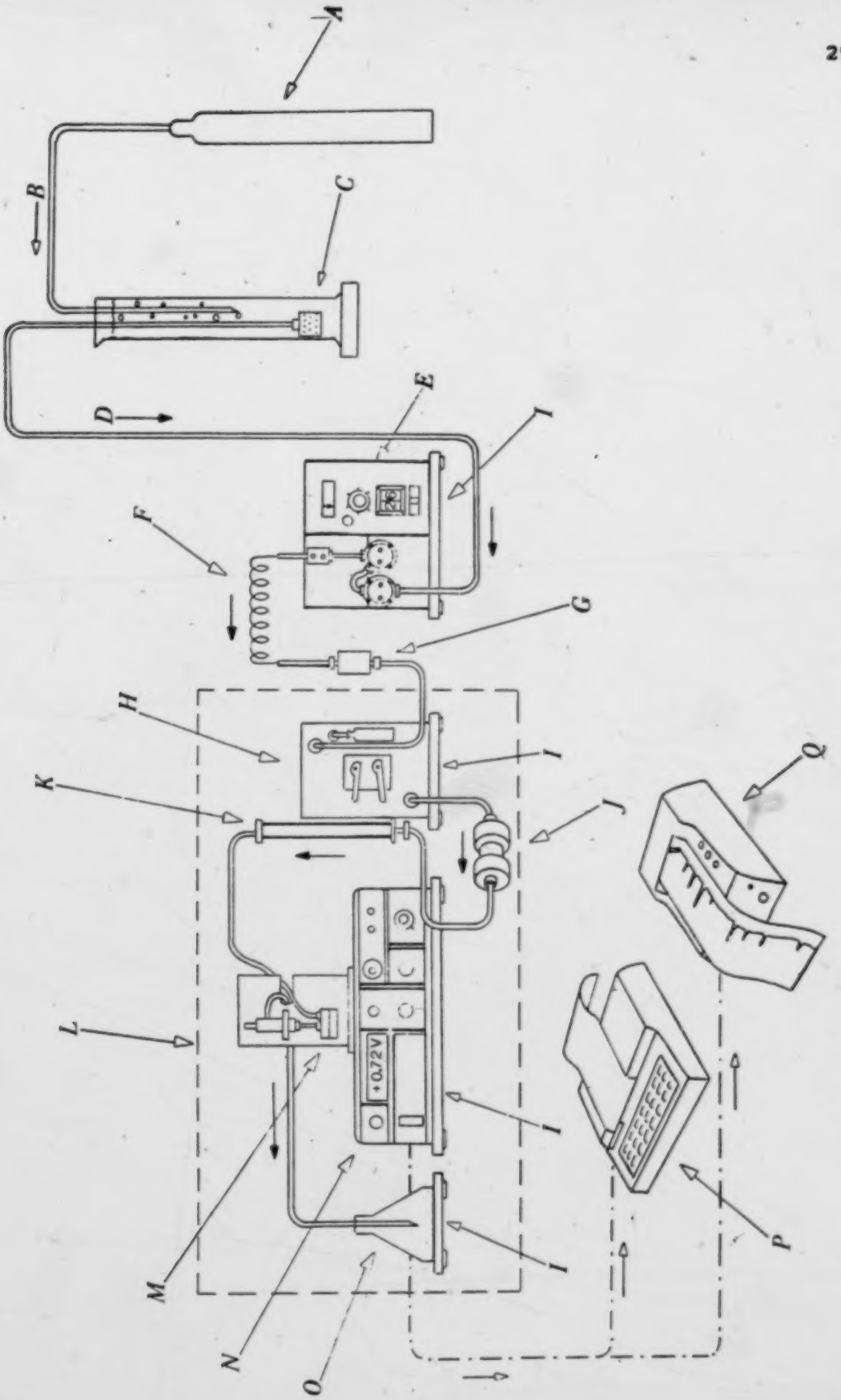
The introduction of electrochemical detectors sensitive enough to detect even resting levels of CA has resulted in

reverse-phase HPLC-ECD becoming the preferred method for analyzing plasma extracts for CA concentration (Figures 3 and 4) [Holly and Makin, 1983; Krstulovic, 1982]. The entire assay (including extraction, chromatography and detection) requires, at most, two hours to complete the analysis of one blood sample. This represents the total amount of time necessary to proceed from a frozen plasma sample to a chromatogram (Figures 5 and 6). Trouble-shooting is thus greatly simplified by the speed of the procedure and the small number of intermediary steps. This offers a significant advantage over previous assay methods (e.g., REA) which required up to two days to complete and in which trouble-shooting was an extremely laborious undertaking.

However, the HPLC-ECD method is not without complications. These include the maintenance of a number of complex instruments which can be capricious. [For a more detailed discussion of the problems associated with the maintenance of HPLC equpiment, the reader is referred to Runser, 1981.] This issue is a particular problem in HPLC-ECD CA assays because the low concentrations of CA normally found in human plasma require the maintenance of a high level of sensitivity of the HPLC-ECD apparatus. Additionally, the HPLC-ECD CA assay relies upon a series of intricate chemical interactions which can also be difficult to control over an extended period of time (see below). To assist the reader in comprehending the intricacies of this assay as emphasized later for application in the clinical laboratory, a short

Figure 3. Schematic drawing of the HPLC-ECD apparatus used in the catecholamine assay, wherein:

HPLC = high pressure liquid chromatography
ECD = electrochemical detector
A = helium gas tank
B = direction of flow of gas
C = mobile phase reservoir
D = direction of flow of mobile phase
E = pump
F = in-line pulse dampener
G = in-line filter
H = injector
I = lucite platform
J = guard column
K = stationary phase (phenyl column)
L = large Faraday cage
M = ECD cell assembly
N = ECD electronic controls
O = waste receptacle
P = Shimadzu integrator
Q = strip chart recorder.


A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
+0,72V

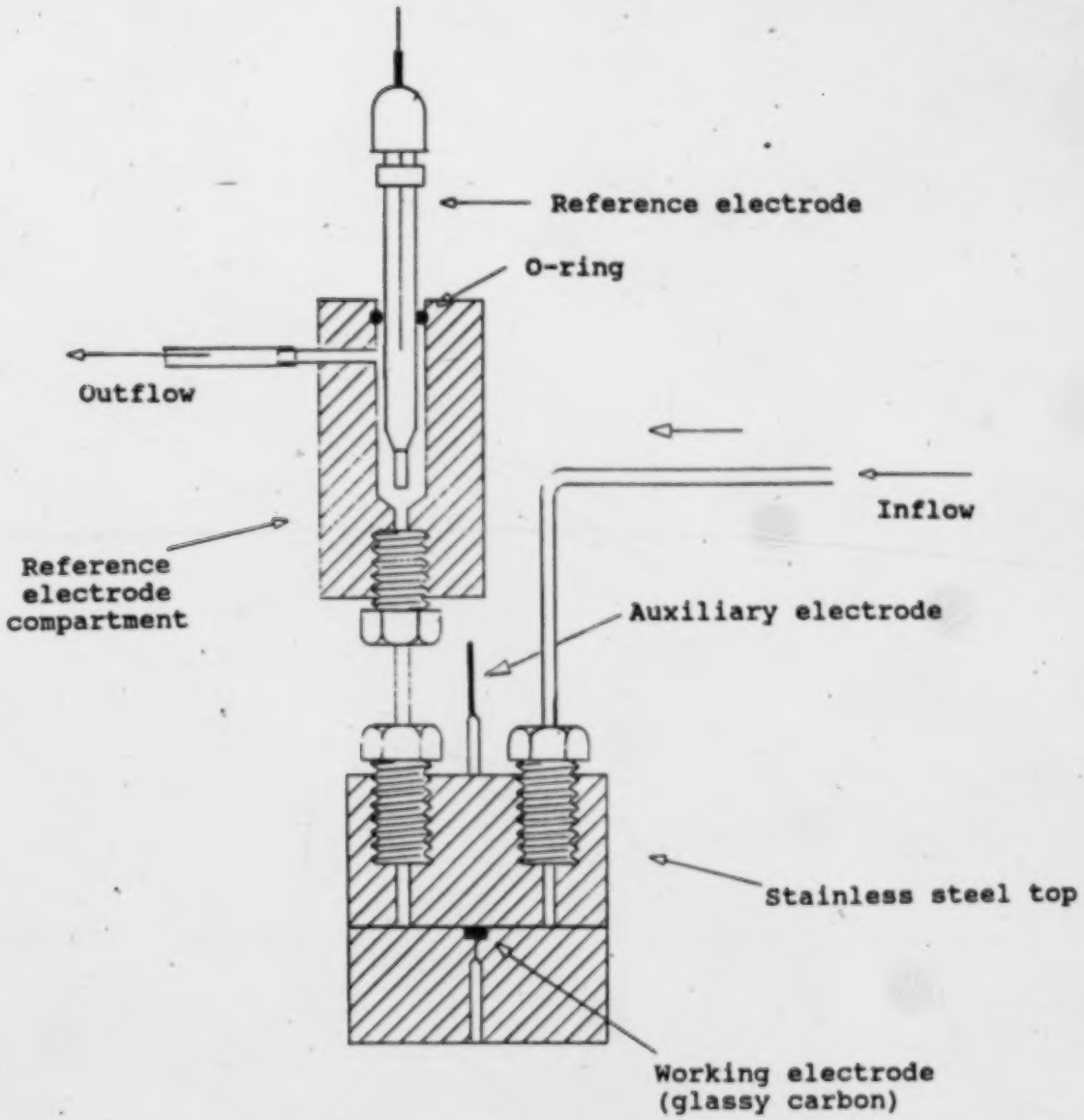
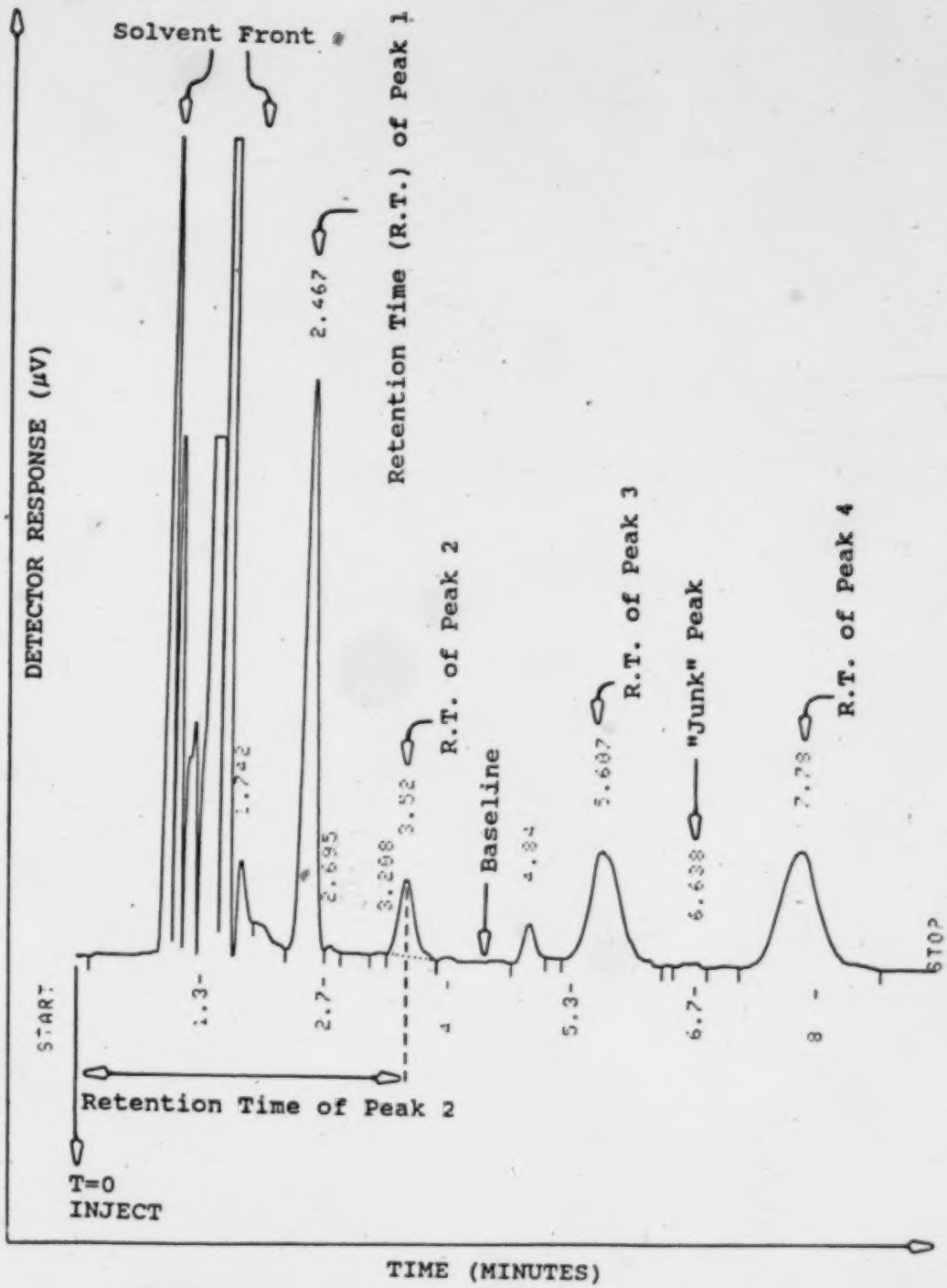


Figure 4. Schematic diagram of ECD cell.

Figure 5. Generic chromatogram generated by HPLC-ECD apparatus of Figure 3, wherein:
Peak 1 = noradrenaline
Peak 2 = adrenaline
Peak 3 = dopamine
Peak 4 = deoxyepinephrine.


Solvent Front
Retention Time (R.T.) of Peak 1
2.467
DETECTOR RESPONSE (μV)
R.T. of Peak 2
3.52
3.258
2.695
Baseline
4.84
R.T. of Peak 3
5.607
"Junk" Peak
6.638
R.T. of Peak 4
7.78
START
1.3-
2.7-
4 -
5.3-
6.7-
8 -
Retention Time of Peak 2
T=0
INJECT
TIME (MINUTES)

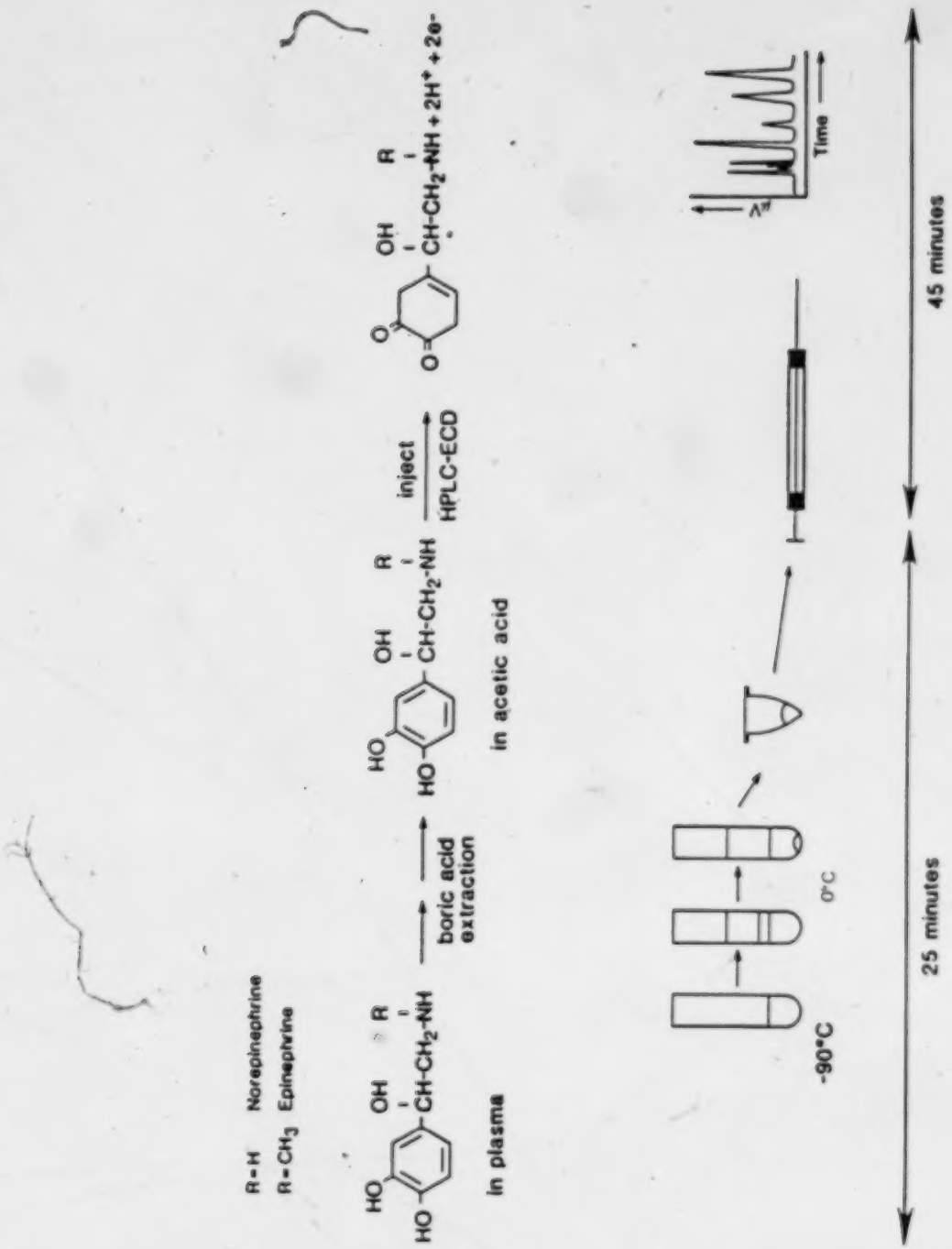


Figure 6. Summary of HPLC-ECD catecholamine assay with boric acid extraction of plasma.

review of the main elements of this CA assay will be presented. The reader is referred to Appendices II & III for a more detailed outline of the underlying principles of the assay. The main elements of the HPLC-ECD assay are extraction of CA from plasma (chemical process), separation of the CA from each other (by HPLC), and, detection and objective quantification of the individual compounds (by ECD and an integrator, respectively) [See Figure 6].

(a) Extraction of CA from Physiological Fluids

Quantification of plasma CA in an HPLC-ECD system requires that the CA first be extracted and concentrated from plasma. Producing as "clean" a sample for injection as possible is important because this part of the assay often determines the ultimate success of the entire procedure. Desirable properties of a good extraction technique include specificity for the analyte(s) of interest and removal of interfering compounds (this not only prevents overloading of the column but also assists resolution of the analytes by the column), concentration of the sample into as small a volume as is practical, and, transferring the analytes into the most appropriate carrier solution in preparation for separation and detection by the HPLC-ECD system.

In CA analysis, the options for removing and concentrating the CA in plasma include activated alumina (effectively, a precipitation technique) [Anton & Sayre, 1962] and a boric acid extraction technique (a chemical extraction) [Smedes et al., 1982]. The chronic problems with either of

these alternatives are specificity, efficiency and reproducibility. Additionally, the extraction technique must deliver the sample to the separation step of the assay (i.e., the HPLC-ECD system) in an appropriate solvent. In practice, the development of an ideal extraction method has proven to be an ambitious goal.

Activated alumina was first used in CA assays in the early 1960's [Anton & Sayre, 1962]. The idea behind the method is that activated alumina particles will differentially adsorb CA molecules (from physiological fluid samples) which can then be eluted from the alumina. In practice, while the procedure is very brief, it is not very successful. First, alumina will bind many things from plasma besides CA. The exact physicochemical basis for this attraction is neither known nor controllable. Secondly, the efficiency of the technique is very low, viz., only 40 to 60% of the CA present in the plasma will actually be extracted. Thirdly, the CA must be eluted from the alumina by the use of perchloric acid; this imposes a limitation on the chromatographic separation conditions to only those which can tolerate the acid.

The reproduciblity of the alumina extraction technique is among its worst problems: batches of alumina vary markedly in their efficiency. The material itself is very capricious and difficult to handle uniformly and there is a good deal of "folk-lore" associated with the treatment methods for alumina. Consequently, inter-laboratory differences in techniques exist and results obtained using alumina vary considerably.

Numerous attempts have been made to solve these problems of inconsistency with the result that additional complexities were created. For example, the alumina extraction has been coupled to a radioenzymatic assay in the hope that the recovered yield could be improved. However, alumina is not an inert material: it inhibits COMT (the enzyme used in most REA's) thereby reducing the apparent yield from the assay [Gauchy, Tassin et al, 1976].

The chemical extraction of the CA from physiological fluids using boric acid was investigated in the late 1970's as an alternative to precipitation of CA with alumina [Molnar & Horvath, 1976, 1978; Horvath et al., 1976]. The method involves the extraction of the CA from plasma in an organic solvent by the use of ion-pair formation with diphenylborate. It is simple to perform and requires only about one half-hour to proceed to completion. The recovery of the CA from plasma is very high (80 to 100%) and the method is very reproducible [Smedes et al., 1982]. Inter- and intra-assay variability of the boric acid extraction can be monitored and standardized by adding a known amount of an internal standard to the plasma prior to the extraction.

(b) HPLC Separation of the CA

Once the CAs have been extracted and concentrated from the plasma, they must then be separated from each other before they can be quantified. This is accomplished by the HPLC apparatus. Normally, there are at least six components to the HPLC system: solvent (mobile phase) reservoir(s), pump,

injector (i.e., a means by which to introduce the sample to be analyzed into the system), chromatography column (containing the stationary phase), detector, and data recorder(s) [see Figure 3] [Bender, 1987]. Each of these components will be described in turn.

The solvent reservoir contains the special mobile phase (MP) which has been selected for optimal separation of the compounds of interest. The composition of the MP will also be determined by the choice of column which, in turn, is determined by the physicochemical properties of the compounds to be isolated from one another and by the choice of detector(s). Thus, no component can be varied without consideration of the HPLC-ECD system as a whole. Because of the sensitivity scale at which the HPLC-ECD apparatus operates, it is extraordinarily sensitive to dissolved gases and particulate matter. This creates a need for high-purity reagents, solvents and water in the MP and for saturation of the MP with an inert gas. The flowpath of the MP is equipped with a variety of microscopic filters designed to protect the column and detector further downstream. Additionally, before the MP enters the HPLC system, it is membrane-filtered and degassed.

The pump must be able to deliver constant, reproducible, pulseless flow of MP (from the reservoir) through the entire HPLC-ECD system. Therefore, the usual practice is to have a double, reciprocating pump (that is, with two pump heads) and to install a pulse dampener(s). The dependability of the unit

itself is usually determined by its manufacturer and there are many good machines to choose from today. It must be emphasized, however, that these pumps represent relatively new technology and, as such, require special handling and maintenance.

For example, because the system is closed and running at high pressure, it must be maintained free of leaks; leaks disrupt flow through the system and are manifested as baseline noise. Additionally, these pumps are miniaturized for the purpose of pumping fluids of variable viscosities through very narrow bore stainless steel tubing capable of withstanding pressures as high as 6000 psi. [N.B., The HPLC columns cannot withstand more than 3500 psi before sustaining damage to the chromatographic bed.] All materials inside the pump which come into contact with the MP must be completely inert and are made from special steel alloys and Teflon. The valves and pistons present a special challenge: not only must they be chemically inert but they must also be able to withstand the rigors of sustained function at high pressure. The valve parts (for example, the ball and seat) are made of synthetic ruby and sapphire, respectively, and the piston rod is sapphire.

The injector is the component which gives the operator access to the column, and thus must be able to deliver the entire sample to be injected (i.e., the mixture of compounds to be separated) to the column without losses of sample or addition of air. Again, as with the pumps, the technology

embodied in these units has improved dramatically in recent years and injectors have become quite dependable. The improved technology is directly responsible for making this the simplest part of the HPLC system to maintain.

The sample mixture to be analyzed is introduced by the injector into the flowstream of the MP to be separated into its constituents by the column, preferably as completely as possible. Therefore, the column is the final determinant of the success and extent of separation of the compounds in the mixture to be analyzed. In an effort to protect the column, a pre-column (called a guard-column, or -filter) is often placed before the main column as a precaution against extraneous materials (e.g., proteins) causing degradation of the packing material and/or loss of resolution.

The choice of columns is determined by the physicochemical properties of the CA. Separation of the CA is on the basis of hydrophobic interactions with the chromatographic bed (or "stationary phase"). To date, the only stationary phases which have proven effective are reverse-phase, irregular, 10μ (or less) silica-based, bonded-C8, bonded-C18 and bonded-phenyl packings. The phenyl packings are ideally suited for CA separation from a theoretical point of view but have previously not been used widely for this purpose.

However, the CA molecules are bases (their metabolites are acids) and have ionic functional groups which render them hydrophilic. Thus, they are not retained by the non-polar

reverse-phase columns. It is possible to circumvent this problem by adding a suitable, large, organic counterion to the MP to form an ion-pair with these functional groups of the CA [Eksborg et al., 1973; Eksborg & Schill, 1973]. This is called "paired-ion partition chromatography". More specifically, the CA molecules are encouraged to form ion-pairs with a counterion in an aqueous mixture set at a pH value which will maximize this tendency.

The counterion (that is, an ion of the opposite charge to the CA) is lipophilic and interacts with the column thereby indirectly increasing the interaction of the CA with the column and allowing separation to take place. This interaction can be modulated by alterations in the concentration and structure of the counterion chosen. For basic compounds, such as the CA, an alkyl sulfonate (e.g., a salt of heptane sulfonic acid) is used as a counterion [Moyer & Jiang, 1978; Ghanekar & Das Gupta, 1978]. While the exact physicochemical basis for the interaction between the CA, the column and the ion-pairing agent is not known [Krstulovic, 1982], the single most important aspect involved in the reaction is that both the CA and the ion-pair reagent molecules must be ionized. (This latter characteristic makes possible the formation of the ion-pair-CA complexes and hence the retention of the CA by the column.) The ionization of the CA and counterion molecules is made possible by the MP, which must be composed of an aqueous buffer solution so as to maintain an appropriate pH.

Fine control of the retention of the CA-counterion pair is then achieved by the addition of an organic solvent to the mobile phase which counter-balances the retention by the column induced by the counterion. Most often, this translates into a small percentage of the MP being composed of acetonitrile or methanol.

Another important issue in the choice of MP composition is that the CA are very sensitive to heavy metal ions; these ions will chelate CA in solution and enhance their decomposition. The MP's which are normally used for separation of CA are acidic and, therefore, relatively corrosive in the HPLC machinery. Therefore, the MP usually contains a chelator of heavy metal ions (such as EGTA [ethylenebis-(oxyethylenenitrilo)-tetraacetic acid] or EDTA [(ethylenediamine)-tetraacetic acid]). In addition to any benefits accrued to the CA molecules, the presence of EDTA in the MP will also reduce the widening (spreading) of the solvent peak (front) on the chromatogram.

(c) Detection and Quantification of CA

Once the compounds of interest have been separated from each other, they are detected [and thus quantified] by a detector positioned downstream from the column. A variety of detectors is encountered in biomedical analytical systems and includes spectrophotometric, fluorometric and electrochemical detectors (ECD).

The fluorometric detectors have proven to be of considerable value in clinical chemistry but their utility in

CA assays has, so far, been severely limited for many of the same reasons previously discussed in conjunction with the fluorometric CA assay. Even though HPLC offers the detector a more highly purified analate than was possible with previous isolation techniques, the problems of cross-fluorescence by interfering compounds, sensitivity and reproducibility remain [Schleicher et al., 1983]. Selective derivatization to strongly fluorescing compounds either pre-, or post-column, can overcome some of these problems but adds others. These include the fact that derivatization complicates the analytical procedure considerably and introduces another potential source of error into the assay procedure. Another problem is that any oxygen remaining in the MP that is introduced into the HPLC system may actually quench the fluorescence of the derivatized compounds.

Recently, laser technology has become available in the field of fluorescence detection. It is expected that lasers will also be available for the detection of ultraviolet absorbance. However, the utility of either of these detection modes in the quantification of human plasma CA remains to be established [Kamperman & Kraak, 1985].

Electrochemical detectors (ECDs) offer a practical solution to the problem of detecting very low concentrations of electroactive, physiologically significant compounds [Kissinger et al., 1981]. ECDs suitable for biomedical applications became available in the late 1970's. These detectors operate on the principle of the electrical effects

of chemical reactions. [For a more detailed discussion of the principles of electrochemistry at solid electrodes, the reader is referred to Adams, 1969.] Electrochemical reaction(s) involve(s) the direct conversion of chemical information into an electrical signal. The electrical current thus produced is directly proportional to the quantity of molecules passing over the detector's working electrode. [See Appendix III for a more detailed description of the principles of electrochemistry.] Figure 4 is a schematic representation of the main components of an ECD cell.

CA readily form o-quinones under the influence of an applied voltage [Sternson et al, 1973]. Because of the level of sensitivity possible with the new electrochemical detectors and, therefore, the freedom from the need to derivatize the CA prior to their quantification, this has become the favored detection mode to use in HPLC CA assays. The actual detection limits of the electrochemical cell are determined by, among other things, the chromatographic efficiency, the detector's conversion efficiency and the baseline noise. Unfortunately, these parameters are not independent and many factors determine the extent of the utility of the ECD in CA assays.

The use of an ECD imposes a variety of restrictions on the HPLC system. For example, the ECD requires a conductive MP and hence, the organic solvent MPs used in normal-phase chromatography are not compatible with this detection mode; therefore, reverse-phase columns are the only ones which are suitable for this detector. Furthermore, the actual

composition of the working electrode (WE) must also be compatible with the MP; for example, a WE made of carbon paste would be damaged by acetonitrile in the MP.

To quantify and record the output signal from the detector, a data recorder(s) is placed downstream from it. An HPLC-ECD system produces data as a variable response over time and is most easily represented in a Y (μV) vs. X (time) format. As described more fully in Appendix II, the actual time required for an individual compound to pass through the chromatographic system is referred to as its retention time. In physical terms, this is the time between injection of the sample and its passage over the WE of the ECD. The response of the ECD to the analytes is displayed on the recording device as a sequential series of peaks comprising the chromatogram [See Figure 5]. The magnitude of the ECD's response to the molecules flowing over the WE is directly proportional to the quantity of the molecules present in the injected sample.

Traditionally, the data recorder(s) consisted of a simple strip chart recorder but, more recently, dedicated integrators have become available. These integrators permit the signal to be recorded, stored and re-analyzed as required and offer more objective analysis of the output signal. However, the accuracy and reproducibility of the data produced by the integrator depends entirely on the accuracy and precision of the chromatography [Halasz, 1980].

In summary, it is clear that boric acid extraction coupled with HPLC-ECD technology (in the reverse-phase, ion-pair mode) are the method of choice for the extraction, separation and detection of concentrations of CA in human plasma at this time. Also, it is apparent that such an assay would have to be optimized towards the low concentrations of CA normally found in this matrix. Any analytical method must be proven to be effective by the performance of specific tests which evaluate the assay technique. A number of criteria, including sensitivity, recovery, reliability and reproducibility, must be met in a standardized fashion before the method can be considered valid and deemed useful [Koch, 1987]. However, once validated, it is equally important that the method be tested using physiologically relevant samples [Gelpi, 1987]. All of these criteria must be established before an assay method is used to analyze unknown samples for the analyte(s) of interest.

H. CA and Subarachnoid Hemorrhage

Stroke, or cerebrovascular accident, is the third leading cause of death in North America [Fox 1983]; about 10% of all strokes are actually subarachnoid hemorrhages (SAH) [Sahs et al., 1969]. Furthermore, over 50% of SAH cases are due to a ruptured intracranial aneurysm which results in blood being present in the subarachnoid spaces [Locksley, 1966]. Whole blood, as well as many of the substances it contains [White, 1983; Cook, 1984], has potent vasoconstrictor activity on

cerebral arteries but, while 40-60% of SAH patients progress into cerebral artery vasospasm [Chyatte & Sundt, 1984], this vasospasm is of unknown etiology [Wilkins, 1986]. Neither is the timing of vasospasm predictable: it can occur anytime from day-3 to day-10 post-hemorrhage [Weir et al., 1978]. However, the occurrence of vasospasm is the single most important cause of the morbidity and delayed neurologic deterioration in survivors of acute SAH. A large number of creative attempts at treatment or prevention of vasospasm have so far been unsuccessful [Wilkins, 1980, 1986].

It has been shown that isolated human cerebral arteries are reactive to administered vasoactive substances such as CA and the response to CA is vasoconstriction [Allen et al., 1976]. Predictably, wide inter-individual differences in responsiveness are observed [Brandt et al., 1981; 1983]. Equally intriguing is the fact that cerebral arteries possess adrenergic innervation which has been shown to be altered after SAH [Duckles et al., 1977; Tsukahara et al., 1986].

NA is present throughout the brain and spinal cord and those brain regions which are richest in NA and noradrenergic nerve terminals are located adjacent to the cerebrospinal fluid (CSF) [Struthers & Dollery, 1985]. Plasma CA levels have been shown to be correlated with CSF levels of CA [Ziegler et al., 1977] and therefore plasma CA concentrations would seem to be a useful "index" of sympathetic nervous system activity after SAH. However, because of the difficulty of measuring CA concentrations in body fluids (above), the CA

hypothesis of SAH has been set aside awaiting improvements in the technology available for the CA assay. The few studies that have been done in this area were not properly controlled nor systematic in their collection of data [e.g., Shigeno et al., 1982]. One of the most frequently encountered shortcomings of the studies performed to date is the lack of adequate control for the individual variation in CA pattern and SNS function [e.g., Yoshida et al., 1987]. If plasma CA patterns are to be used as indicators of central SNS activity, it is imperative that individuals act as their own controls in these studies [Holly & Makin, 1983]. Therefore, work, to date, has yielded no definitive answers regarding the role of CA in vasospasm after SAH except to confirm the suspicion that they might be involved.

To address the question of a possible role for CA in vasospasm after SAH, the following study was designed in which plasma CA levels were measured in patients who had suffered a SAH due to a ruptured cerebral artery aneurysm. Plasma samples were taken periodically over the course of the study with the first sample from as close to the time of the hemorrhage as possible (in practical terms, this was the time of admission to hospital), through the time of the possible development of vasospasm and to the resolution of the problem (up to three weeks later).

I. Objectives of the Research Study

The primary objective of the research study undertaken and described herein was to create a reliable, reproducible assay for human plasma CA, insofar as current technology permitted. The rationale for this was that if a newly available technology was to be used in physiological studies, the methodology had to be reliable. This has previously not been the case. It was proposed that an HPLC-ECD system combined with boric acid extraction could be used to accurately quantify human plasma CA. The specific goal of the CA assay procedures described herein was to create conditions that were conducive to the ability of the HPLC-ECD apparatus to hold a (high) sensitivity level of 1 nA full-scale deflection (F.S.D.) of the ECD output over a prolonged period of time.

Secondly, it was desired to attempt to use this assay under the real-life constraints of a clinical study for the purpose of attempting to solve a clinical puzzle. Namely, it was proposed to determine whether plasma CA levels were elevated in patients who had suffered a SAH due to rupture of a cerebral artery aneurysm. And, if so, it was to be determined whether this elevation in plasma CA was associated with the development of cerebral artery vasospasm. The hypothesis was that patients progressing to vasospasm would exhibit elevated plasma CA concentrations just prior to spasm.

METHODS

A. Catecholamine Assay

1. Chemicals

All of the chemicals which ultimately came into contact with the HPLC-ECD system, either directly or indirectly, had to be of the highest available purity. That is, chemicals, reagents and solvents had to be at least Reagent Grade and preferably HPLC-Grade (with a level of impurity no greater than 10 ppm). The water which was the basis for the mobile phase and all of the solutions involved in creating the mobile phase and extraction solutions had to be Type I Reagent Grade Water with a resistivity of at least 10 MΩ (and preferably better than 18 MΩ) [NCCLS Document C3-P2, 1985; ASTM Standards Manual, 1987].

The sodium phosphates (i.e., mono-, and di-sodium phosphate), EDTA, ammonium chloride and heptane were from BDH Canada Ltd., Toronto, Ontario (Assured ACS Grade). The methanol was OMNISOLV-Grade and also from BDH Canada. The acetic acid was Aristar Grade from BDH, Poole, England. The ammonium hydroxide was from Fisher Scientific Ltd. (Ottawa, Ontario), Fisher Reagent ACS Grade. All other commonly used acids, bases and salts were also from Fisher.

Diphenylborate ethanolamine [DPBEA] of the required level of purity was commercially available from Aldrich Chemical Co., Milwaukee, WI.

Tetra-octyl ammonium bromide (TOABr) of the required level of purity was purchased from Fluka Chemical Corp., Ronkonkoma, N.Y.

CA for use as standards were purchased from Calbiochem-Behring [Division of American Hoechst Corporation, La Jolla, CA]. All standard solutions of CA were made up in cold 80 mM acetic acid (ultrapure solution, membrane-filtered) and diluted and stored in cold, amber glass bottles. These solutions were only stable for 48 hours. DOE was purchased from Sigma Chemical Co., St Louis, MO.

[Aside: It is the convention in the CA literature to refer to the concentration of solutions containing CA in terms of pg/mL of solution rather than SI Units which are expressed as molar quantities. If the corresponding SI Unit values are required, the values in pg/mL must be divided by the molecular weights of the CA, namely: 169.18 (NA), 183.20 (A) and 153.18 (DA).]

Octanol of the required level of purity was not commercially available. Therefore, octanol with as high a level of purity as is available [i.e., BDH G.P.R. Grade n-octanol, BDH, Poole, England] was purchased and purified further in the laboratory. The procedure was as follows: in a separatory funnel in a 1:4 (v/v) ratio of wash solution:octanol (typically 50 mL:200 mL, wash:octanol), the following sequence of wash solutions was shaken, three times, consecutively, with the octanol for about two minutes at each step; the phases were allowed to separate from each other, and

the aqueous phase was discarded each time: 1 N sodium hydroxide, ultrapure water, 1 N nitric acid, ultrapure water. At the end of the procedure, the octanol was filtered through paper to remove any remaining traces of the washing solutions. This procedure required one day to proceed to completion and the resulting octanol was stable for up to eight months, at room temperature, in a clean amber glass bottle.

The sodium hydroxide and nitric acid solutions that were used above were made from at least Reagent Grade compounds (Fisher) and diluted in Type I water.

2. Equipment

The HPLC-ECD apparatus assembly included (in series): a tank of inert gas; a reservoir for mobile phase; a high pressure, dual piston, reciprocating pump; an injector; a column; an electrochemical detector; data recorders; and a waste facility [See Figure 3]. For this study, these components were, respectively, a tank of helium (dry), a mobile phase reservoir (graduated cylinder) that could accommodate both the helium gas bubbling in and an in-line filter for the in-take of the mobile phase, a Waters [Waters Chromatography Division, Milford, MA] M-45 pump, a Waters U6K injector, a 30 cm long, Waters, μBondapak phenyl column, a BAS (Lafayette, IN) LC-4B Electrochemical Detector, a Shimadzu [Columbia, WA] C-R3A dedicated integrator and a Linear [Reno, NV] M55 Strip Chart Recorder [N.B.: these data recorders were connected in parallel], and an Erlenmeyer flask as the waste receptacle.

For the purposes of the CA assay, the HPLC-ECD apparatus was outfitted with a variety of special modifications. These accessories facilitated sustained functioning at a high level of sensitivity (e.g., 1 nA F.S.D.) of the ECD and included the following: additional in-line filters and pulse dampeners; a guard column [a 1-cm long column containing (reverse-phase) microparticulate, silica-bonded C_{18} (Waters)]; a stainless steel top on the ECD's working electrode assembly; modified ECD electronics [the modifications including installation of 3-pole Butterworth filters instead of the traditional low-pass RC-filters]; a large Faraday Cage was constructed around the injector, column, detector and waste flask and each one of these components was isolated from the cage by a lucite platform; each component of the apparatus was isolated electrically by being powered on individual power circuits and by grounding with shielded cables; all components of the HPLC-ECD were grounded to a common ground point; and finally, the HPLC-ECD apparatus was powered by a protected power circuit. The HPLC-ECD apparatus produced a more stable baseline signal when the ambient temperature in the room housing the apparatus was between 21°C and 23°C with a humidity level of 30-60%. Similarly, the laboratory was furnished with incandescent, as opposed to fluorescent, lighting in order to further stabilize the output signal from the ECD.

3. Preparation of Glassware

The care and cleanliness of the glassware was deemed essential to the success of the CA assay and as such, is

described herein. The following procedures were performed on all glassware which ultimately came into contact with the chemicals used in the assay and/or attendant equipment (including the HPLC-ECD apparatus - described below). This included all glassware used to prepare the mobile phase and its constituents and the components of the extraction procedure (e.g., Erlenmeyer flasks, graduated cylinders, bottles, etc.).

First, and prior to initial use of the glassware in the CA laboratory, the glassware was subjected to a dichromic-sulfuric acid bath. The glassware was soaked in the acid solution overnight and the acid was subsequently rinsed away with copious quantities of distilled water. The glassware was then washed in a solution of a substantially residue-free detergent, such as Hemo-Sol (Fisher), diluted in water (preferably distilled water). The glassware was soaked in the detergent solution for at least two hours, preferably overnight. The detergent was rinsed away with copious quantities of distilled water; a final rinse was performed using double-distilled or de-ionized water before the glassware was oven-dried. Once the glassware had dried, it was stored with all orifices covered.

Each piece of glassware was used only once between washings and after each use, it was placed back into the detergent washing solution prior to undergoing the above washing procedure again. The dichromic-sulfuric acid bath

treatment was repeated only as required (i.e., when a residue had built up on the contact surface of the glassware).

Any glassware which was used to extract the plasma (i.e., the CA-containing matrix) or to transfer or to store it had to be siliconized prior to use because CA adhere to bare glass. Among the glassware which was siliconized were the culture tubes (in which the extractions were carried out) and the transfer, glass Pasteur pipettes. A siliconizing solution was prepared into which clean glassware (directly from the oven after washing as described above) was dipped momentarily and then the glassware was baked in a high-temperature (200°C) oven. The siliconizing solution was either a commercially prepared mixture (Serva Feinbiochemica, Heidelberg, W. Ger.) with the manufacturers' directions followed for the preparation of the glassware or, alternatively, was a 5% solution of dichlorodimethylsilane (obtainable from Matheson, Coleman & Bell, New York, N.Y.) in HPLC-grade toluene (Fisher). In the latter case, the clean glassware was immersed in the siliconizing solution, the excess allowed to drain and the glassware baked in a 200°C oven for two hours. The siliconized glassware was rinsed with double-distilled water after the baking treatment to remove any excess silane and then oven-dried. After the glassware had cooled, it was stored covered to protect it from dust.

If too much silicone had been deposited on the contact surface of the glassware, that surface appeared slightly cloudy but the glassware was still usable in that condition.

The integrity of the silicone-layer coating was ascertained from the appearance of the glass: if well-defined beads of water formed on the silicone-coated surfaces, it was assumed that the monolayer had not been damaged by use. If water did not form discrete beads on the coated surface, the item was re-siliconized. Normally, the culture tubes were re-siliconized after each use due to the corrosive effects of the ammonium hydroxide-ammonium chloride solution used in the extraction procedure (below). In this case, tubes were washed as above, dried and re-siliconized. It was not necessary to remove the remnants of the silicone coating between treatments.

4. Preparation and Care of HPLC-ECD Apparatus

(a) Before Operation for the First Time

The HPLC-ECD apparatus arrived from the manufacturer with a solvent (methanol) in the flowpath. While the apparatus was being connected, and later, when repairs were made, methanol was pumped through it to keep air from collecting in the apparatus and to flush away any contaminants. Care was taken that all of the compression fittings on the apparatus were made so that they were leak-free. Similarly, all of the connections on the apparatus must be tight-fitting. Any and all solutions which ultimately came into contact with the apparatus were filtered through a 0.22 μ pore-size membrane filter and de-gassed (in a special glass, filtration apparatus designed specifically for this purpose

[Millipore Corporation, Bedford, MA]) for at least 20 minutes prior to use in the apparatus.

Once the HPLC-ECD apparatus had been assembled, it was purged with nitric acid before use in the CA assay. It was essential that the column be disconnected from the system during this nitric acid wash. [The detector cell compartment is also normally not subjected to the nitric acid treatment unless there is some special reason for which it might require this.] About 100 mL of the following solutions were passed through the apparatus, in the designated order: pure methanol was replaced by 50:50 (v/v) methanol:water, to pure water, and then 6 N nitric acid. Then the nitric acid was flushed out of the apparatus using water until the effluent had returned to neutral pH.

Once the nitric acid had been washed completely away, the apparatus was flushed with 100-150 mL of the following solvents, in the designated sequence: pure water, to 50:50 (v/v) methanol:water, to pure methanol. The column and detector were re-connected to the rest of the apparatus and the entire assembly was flushed with pure methanol. It was then flushed with the following sequence of solutions (about 150 mL each): pure methanol, to 50:50 (v/v) methanol:water, to pure water [at this point, the reference electrode was replaced into the housing] and then the MP (described below) was introduced into the system. Once the system had been brought to MP, it was run at 500 psi for approximately 72 hours to attain an equilibrium of the electrochemical detector

cell at 1 nA full scale deflection (F.S.D.). [Alternatively, the system could be run at 2000 psi in the daytime and 500 psi overnight for 48 hours to attain equilibrium.]

(b) Preparation of Mobile Phase

The optimal MP, as determined for the detection of the CA, was composed of 70 mM sodium phosphate buffer, pH 4.8 at 22°C, containing 5 mM heptanesulfonate (HSA), 1 mM ethylenediamine tetraacetic acid (EDTA) and 5% (v/v) methanol. [This solution is sometimes referred to herein as the "daytime mobile phase".] The complete MP is freshly made immediately prior to use and then filtered through an 0.22 μ pore-size membrane [Millipore GVWP 04700] and de-gassed by vacuum with stirring (for at least 20 minutes) prior to introduction into the HPLC-ECD system. The pump was set to give a MP flow of 2.4 to 2.8 mL/min.

(c) Daily Maintenance of Apparatus

At the end of every day of operation of the HPLC-ECD apparatus, the system was flushed ("scrubbed") overnight with one of two possible MPs: either a 70 mM sodium phosphate buffer, pH 4.8 at 22°C, containing 5 mM HSA and 15% (v/v) methanol or a 70 mM sodium phosphate, pH 4.8 at 22°C, buffer with 0.5 mM HSA, 1 mM EDTA and 15% (v/v) methanol. The choice of the two options was determined by the type of samples that were injected on that particular day. If CA standards were the principal samples injected on the day in question, then the latter overnight MP was used; if the principal samples injected were extracts of plasma, then the former solution was

used. The overnight MP was pumped through the system with a pressure head of about 500 psi (therefore, at a flow rate of about 0.5 mL/min). Mobile phases were not re-cycled.

(d) Weekly Maintenance of Apparatus

At the end of every week, an abbreviated version of the methanol wash sequence described above was flushed through the pump and injector. In this case, only 50 mL of each of the following solutions was passed through the system, in the designated order: MP, to pure water, to 50:50 (v/v) methanol:water, to pure methanol, and back through 50:50 (v/v) methanol:water, to pure water, to MP. During this wash, the detector cell was not turned off.

Occassionally, to clean the column and detector cell without loss of equilibrium and sensitivity, repeated injections of 50 µL of pure methanol were made.

(e) Monthly Maintenance

Once every four to six weeks (or, as required) the entire HPLC-ECD apparatus was cleaned by purging it with pure methanol. This was accomplished by changing the running solution from MP to pure water (the ECD was turned off and the reference electrode was taken out at this point and placed in a solution of 3 N NaCl), to 50:50 (v/v) methanol:water, to pure methanol. About 150 mL were passed through the system at each of these steps. All of these solutions were pre-filtered through the 0.22 µ membrane and de-gassed.

It must be noted that running pure methanol through the column was assumed to be the only safe way to try to rid it of

contaminants resistant to the overnight MP (above). If something had been deposited on the column that was not amenable to such manipulation, the column had to be replaced. It is also worthy of note that, for the purposes of the CA assay, these columns functioned at an optimum level for the duration of only about 300 injections; after approximately 400 injections, the columns had to be replaced altogether. For other uses, HPLC columns have been shown to last for the duration of approximately 1200 injections [Scott, 1987, personal communication].

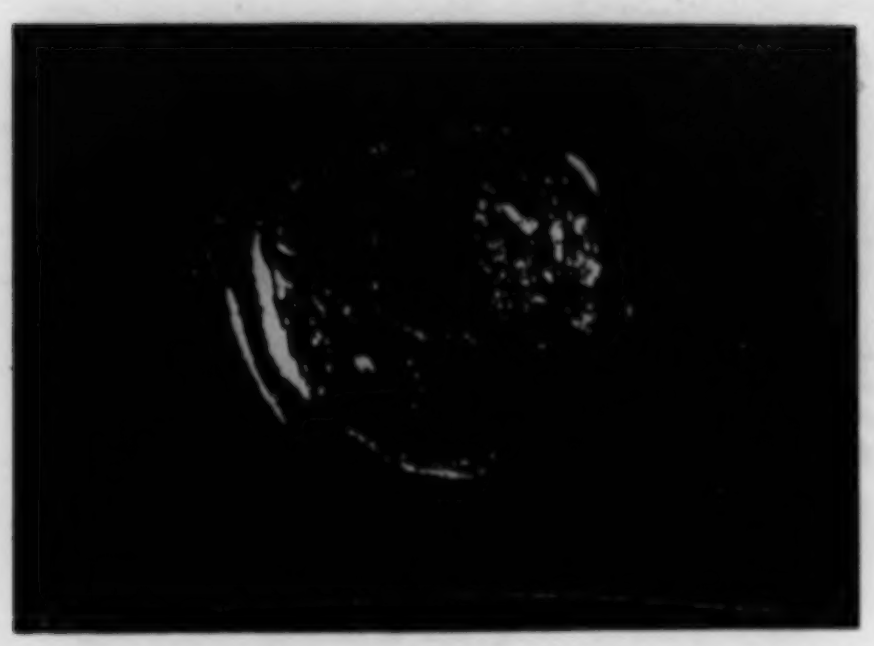
Repairs were made to the HPLC-ECD apparatus only while the flow-through system contained pure methanol. Pump repairs were normally carried out on a monthly or bi-monthly schedule. Whenever the compression fittings or pump heads were repaired, care was taken to ensure that air did not enter the system as a result of the repair procedure. It was also very important to maintain all fittings leak-free. This was accomplished by ensuring that the ends of the tubing were cut perpendicularly [See Figure 7] and the ferrules and compression screws were positioned properly on the tubing. The Guard column was changed (while the system contained methanol) after 50 injections or as required.

The other item to be cleaned on the monthly schedule was the glassy carbon working electrode of the ECD (described below). Once this task had been completed, the apparatus was set to run on MP again by passing the following sequence of solvents or solutions through in the designated order: pure

Figure 7. Photographs of the cut ends of the tubing of the flowpath of the HPLC-ECD apparatus wherein:
A = tubing which has been scored around its circumferance with a file and then broken off
B = tubing which has been scored at two points (on opposite sides of the tube) and then broken off
C = demonstration of the compression of the end of a tube due to incorrect placement of a ferrule; also shows the corrosive effects of the mobile phase
D = presentation of a perfectly cut and polished end of a tube.

A

B



C

D

methanol, to 50:50 (v/v) methanol:water, to pure water (the reference electrode was replaced in its housing), to daytime MP. About 150 mL of each solvent or solution were passed through the system at each stage.

(f) Care of Glassy Carbon Electrode

The criterion for the initiation of the polishing procedure (see below) was derived on the basis of the loss of resolution of the A peak: a 25% reduction in maximum attainable peak size combined with a concomitant increase in baseline noise which further obscured the peak indicated the need to polish the electrode. When plasma samples containing low, resting levels of A were being injected, the above criterion represented a significant, and unacceptable, loss of sensitivity. In practice, the polishing technique described below was necessitated only about every four to six weeks, depending on the types of chromatograms being run; this was due, in part, to the effectiveness of the daily and weekly regimens for the care of the HPLC-ECD system including, especially, the overnight MP.

The method used was as follows: with the HPLC-ECD system containing methanol, the working electrode assembly was taken out of its mounting and then taken apart. [The manufacturer's instructions for doing this were closely followed.] The top part (stainless steel) of the cell and the gasket (Teflon) were rinsed off with ultrapure water then briefly (30 seconds) sonicated in 50:50 (v/v) methanol:water

(both ultrapure reagents) and then rinsed with water and oven-dried.

The bottom half of the cell, containing the glassy carbon electrode, was polished with, in sequence, 6 μ and 0.25 μ diamond fragments. [Obtainable as an aqueous paste from Buehler Ltd., Lake Bluff, IL.] Natural diamond fragments were used but it is also possible to use synthetic diamond fragments. [It is desirable to use 1 μ natural diamond fragments in between the other two sizes of polish but it is not absolutely necessary.] Several drops of water were placed on a circle of diamond polishing cloth (e.g., Microcloth [Buehler Ltd., Lake Bluff, IL.]) onto which 1 or 2 mL of the paste were squeezed. The cell was placed, face down, onto the paste and with gentle pressure of the hand (applying slightly more pressure than would be exerted by the weight of the wrist alone), the cell was rubbed on the cloth for 30 seconds in a clockwise direction and then for 30 seconds in the counterclockwise direction. If the cloth started to have heavy black streaks on it during this procedure, it was an indication that excess force was being applied.

The cell was rinsed with copious quantities of water in between the different sizes of diamond polish (applied in decreasing order of size). At the end of the polishing procedure, the cell was rinsed with water and then sonicated briefly (30 seconds) in 50:50 (v/v) methanol:water, rinsed with water once again and dried in a low-temperature (50°C) oven for about 5 minutes. Care was taken not to allow

fingerprints to be deposited anywhere near the reactive surface of the cell. The cell was replaced in its sandwich configuration (taking care that the four screws which hold the assembly together were tightened equally) and re-mounted in the apparatus. Care was also taken to remove any air bubbles which accumulated while the cell assembly was being re-connected to the system.

The HPLC-ECD system was brought back into mobile phase as described above and the cell was allowed to equilibrate before injections were made.

5. Extraction Procedure

Human plasma was used as the model system but it is emphasized that the following procedures can successfully be carried out on other physiological fluids (including cerebrospinal and extracellular fluids). The procedures described below were carried out in a fume hood. The method of Smedes et al. (1982) was used with the sole modification being the use of DOE as the internal standard instead of 3,4-dihydroxybenzylamine (DHBA). [See Figure 8].

Two mL of plasma were placed into a cold, silicone-lined culture tube (glass and with a teflon-lined screw cap) to which 250 µL of the internal standard solution, DOE, were added (concentration of the standard solution of 4 ng per 50 µL of 80 mM acetic acid). To this mixture was added 1 mL of a 2 M NH_4OH-NH_4Cl buffer containing 0.2% (w/v) diphenylborate ethanolamine (DPBEA) and 0.5% (w/v) EDTA. Finally, 5 mL of an heptane-1% octanol solution containing 0.25% (w/v)

Figure 8. Summary of the chemical principles underlying the boric acid plasma extraction method [Adapted from: Smedes et al, 1982].

tetraoctylammonium bromide (TOABr) were added. The tube was tightly closed and the mixture was shaken for 2 minutes at high speed (e.g., 280 excursions per minute) in a reciprocating utility shaker [Eberbach Corporation, Ann Arbor, MI] fitted with a utility carrier capable of accommodating a rack of culture tubes positioned horizontally. The sample was then centrifuged at 1,200 x g for 5 minutes in a refrigerated (+4°C) centrifuge [IEC, Fisher].

The tube was removed from the centrifuge and the upper organic layer of the contents lifted off using a siliconized, glass Pasteur pipette and transfered into a clean, siliconized culture tube. To this new tube were added 2 mL of pure octanol and 250 µL of an ultrapure, and membrane-filtered, solution of 80 mM acetic acid. The tube was sealed and shaken again at high speed for 2 minutes. The tube was centrifuged in the refrigerated (+4°C) centrifuge for five minutes at 1,200 x g.

The tube was removed from the centrifuge and the upper, organic layer was carefully lifted away from the lower aqueous layer by vacuum suction and discarded. The aqueous phase was transferred into a chilled, small plastic tube (e.g., 1.5 mL Eppendorf micro-centrifuge tube) with a tight-fitting lid, with the aid of a siliconized glass transfer pipette. This small tube contained the sample to be injected into the HPLC-ECD aparatus and this extract was assumed to be stable, on ice, for only 1 hour. It was possible to make 2 injections of the sample during this time but if any more injections were

required, another aliquot of the same sample was assayed. Unknown samples were analyzed in duplicate.

6. ECD Detection and Quantification of CA

Injection volumes for the HPLC-ECD did not exceed 100 µL and normally, 50 µL of plasma extracts (or CA standard solutions) were injected. As mentioned above, a Waters µBɔndapak phenyl column (30 cm long) was used for this assay with a Waters µBondapak C_{18} guard column positioned upstream from it.

The ECD was set at a range of 1 nA F.S.D. with the active filter on maximum filtration. The applied voltage was set at +0.72 V (with the toggle switch on the back panel of the ECD in the OXIDATIVE MODE position). The offset circuitry was set to compensate for the background current. The data recorders were set up, in parallel, from the appropriate output jacks on the back panel of the ECD. The reference electrode was periodically checked against a standard voltage source.

7. Validation of Assay

It was necessary to establish the optimum operating conditions of the apparatus and to test the validity of the assay method. The methods summarized below are unique in that these experiments were all performed at 1 nA F.S.D. (with the exception of the polarogram), demonstrating the ability to achieve and maintain the high sensitivity of the ECD necessary for the CA assays.

The data were recorded in terms of micro-volts (μV), which represented the units as converted, stored and displayed by the Shimadzu integrator (the output from the integrator was derived from the actual voltage generated by each CA as it passed over the ECD electrode). Thus, the integrator provided a more objective assessment of peak height than would be obtained by measuring peaks manually. Additionally, the integrator permited re-calculation and subtraction of peaks because a library of data could be created and stored.

(a) Determination of the Operating Potential of the ECD

The exact voltage to be used in the ECD to obtain maximum oxidation of the CA under the current set of chromatographic conditions had to be determined. It is emphasized that whenever the chromatography conditions were significantly altered, the optimum operating potential of the ECD for the CA also changed. Optimum operating potentials of the ECD cell were determined by performing hydrodynamic voltammographic studies on the compounds of interest, as follows:

CA oxidize to o-quinones under the influence of a positive voltage. The HPLC system was set to run at an intermediate speed (approximately 2 mL/min) and the voltage of the cell was set at +0.20 Volts using a range setting (of the ECD) of 5 nA F.S.D.. A standard injection (e.g., 50 μL) of a mixture of CA, each CA being present in equal proportion (e.g., 1000 pg each of NA, A, DA and DOE), was then made onto the column. At this voltage, there was no ECD response to the

CA and the strip chart recorder (and/or integrator) only revealed a small solvent front peak followed by a flat baseline, indicating that no oxidation of the CA had taken place on the the working electrode surface. The voltage was increased in steady increments of, for example, 0.02 or 0.04 V each time, while repeated injections were made. As the voltage was increased, the current response of the individual CA also increased until a plateau was reached. On the output of the strip chart recorder, there was observed a series of peaks of ever-increasing height as the cell voltage was increased. But, because each of the CA has separate physicochemical properties, the height of these peaks changed at a different rate for each molecule. The procedure was stopped at about +0.90 V. A graphic plot was made of the response of each CA (in terms of current) to the applied voltage and the final plot (polarogram) was constructed.

The operating voltage that was used in the assay was chosen from these data as that voltage which yielded the maximum oxidation of all the compounds of interest.

(b) Composition of the Mobile Phase

The composition of a MP was chosen on the basis of the needs of the column (i.e., the stationary phase aspect of the chromatography conditions), the detector and the physicochemical properties of the analytes of interest. For the CA assay, the buffering ingredient normally used is phosphate, and the 2 other major components which are normally titrated are methanol and an ion-pairing agent such as

heptanesulfonate (HSA). Concentrations of these are adjusted until an acceptable resolution of the analytes (CA) is obtained. In the present study, the 70 mM sodium phosphate concentration was used throughout and experiments were performed varying the concentrations of the HSA and the methanol until acceptable chromatograms were obtained. Based on prior knowledge of the phenyl column [Neill, 1984, personal communication], concentrations of methanol ranging from 0.5 to 25% (v/v) and 0.5 to 5.0 mM HSA were tested. Based on these tests, 1 analytical MP and 2 different scrubbing MPs were selected (as described above in Section 4(c)).

(c) Repeatability of Injections

The following concentrations of CA standards were injected (simultaneously) 5 consecutive times, with the cell equilibrated at 1 nA F.S.D.: 2500 pg of NA; 500 pg of A; 1000 pg of DA; and, 2000 pg of DOE (in 50 µL injections).

(d) Determination of Linearity

A series of solutions of CA standards were prepared representing the physiologically relevant concentration range and injected in triplicate. The important factor was the amount of the substance(s) injected onto the column, and not the concentration of the solution from which that injection came. The quantities injected were:

Picograms Injected in 50 μL

NA	A	DA	DOE
100	20	40	80
250	50	100	200
500	100	200	400
1250	250	500	1000
2500	500	1000	2000
3750	750	1500	3000
5000	1000	2000	4000

A standard curve was plotted of detector response (in μV) versus the amount of CA (in pg).

(e) Determination of Per Cent Recovery

These experiments were performed using CA-free plasma ("blank plasma"). This plasma was spiked with known amounts of CA and then extracted and the CA quantified. The amount of CA used in this experiment was chosen so as to be representative of the physiologically relevent concentration range. The data were calculated as both absolute and relative (i.e., relative to the internal standard) recovery of CA. An abbreviated version of this experiment was run every day by the inclusion of the internal standard in the sample being extracted. It was extremely important to assess the losses of analyte(s) incurred during the running of the assay. The entire experiment was usually performed about twice a year or after any major change or modification to the HPLC-ECD system.

This assessment was performed by extracting triplicate samples of plasma containing one of three concentration ranges of CA representing a high, medium and a

low value from the human physiological range. The concentrations chosen are shown in Table 1.

Table 1. CA concentrations used in recovery experiments

	Picograms Injected in 50 µL		
CA	HIGH	MEDIUM	LOW
NA	5000	2500	500
A	1000	500	100
DA	2000	1000	200
DOE	4000	2000	400

The plasma normally used for this experiment was from a pool maintained in the laboratory and was collected from subjects who were not taking any drugs. Preferably, the population from which this blood is collected remains the same over time. For the majority of the results reported herein, the pool of blood was derived from a single individual, the author.

(f) Inter-Assay and Intra-Assay Variability

The systematic and random error of the assay technique had to be established. The systematic error was assessed by analyzing the same blood sample, spiked with a known quantity of CA, repeatedly on the same day. The medium concentration level set forth above (in the description of the

recovery experiments) was assayed in 5 samples of plasma on the same day.

Inter-assay variability is an estimate of the random error which can be expected to creep into the assay method over a longer period of time. Typically, the unit of time used to evaluate this is 1 week. Once again, the more repetitions that are possible on a single sample the better, and, it would be advantageous to cover the physiologically relevent concentration range. However, such a labour-intensive assessment would be impratical. The compromise position is to select a mid-range concentration level and evaluate its' performance over one week. Therefore, the medium concentration range mentioned above was selected and assayed in triplicate each day for a week.

(g) Stability of Plasma Samples

The stability of the CA in the collected plasma samples must be determined as well as their stability in the frozen state awaiting analysis. CA-free plasma samples were spiked (in duplicate) with a known amount of CA and placed on ice and in the dark. These samples were assayed immediately, after 1 hour, and after 3 hours.

The stability of frozen plasma samples was tested by spiking CA-free plasma with a known quantity of CA, freezing the samples at -90°C [on November 5, 1985] and thawing these samples approximately 6 months [June 25, 1986], 9 months [August 14, 1986] and 20 months [July 20, 1987] later.

B. Human Studies

1. Collection of Plasma Samples

The protocol for the collection of blood samples was established only after consideration of a number of general principles, as follows: it is imperative that subjects participating in CA studies act as their own controls (Holly and Makin, 1983); if a subject is to be followed throughout the course of the evolution of some clinical problem, the subject must have a blood sample drawn at the point of initial contact with the researchers and/or clinicians and at regularly maintained and fixed intervals thereafter; if the subject/patient is to be followed over a period of time (such as (a) week(s)), it is preferable that samples taken during this interval be taken at a fixed time(s) of the day considering the circadian rhythms of the CA. (It is important to avoid the natural, diurnal elevations in plasma CA levels.)

If a subject is to be followed throughout the course of some clinical condition (e.g., after a myocardial infarction or following a SAH or during a psychiatric crisis), it is imperative that a sample of blood is obtained as soon after the onset of the crisis/incident as possible and preferably (if at all possible) before any drugs have been administered. This establishes that individual's "baseline CA state", and progress in the individual's condition is determined relative to this starting point. Thereafter, samples should be obtained at least once a day (preferably twice) at a standardized time (e.g., once in the morning and once in the

evening) and in a standardized fashion until the resolution of the physiological problem or crisis (or within the terms of reference of the study). Collection of samples for assay of CAs is always accompanied by collection of other physiological information (including heart rate and blood pressure) and collection of information regarding nutrition and drugs ingested and/or administered. The subject's level of alertness must also be noted (i.e., a person who is asleep is not equivalent, in a CA sense, to a person who is awake).

Collection of plasma was always performed according to the following protocol: the fasted subject was fitted with an indwelling catheter (size 18 needle, or less, was preferred) connected via flexible tubing and a 3-way stopcock to an intravenous (i.v.) physiological saline solution. The i.v. drip was set to run at a slow speed (such as 0.4 mL/min). A peripheral vein, such as the antecubital, was the most common sampling site but other veins and arteries are also suitable. Whatever the site of this line, in the body, it was kept constant throughout the time of the study. [If a patient was being monitored throughout a hospital stay and progessed from an arterial to a venous line, a sample of blood was obtained from both lines on the day that the cross-over was made.]

After the i.v. line was established, the subject was left alone to sit quietly, without stimulation (this included conversations and reading) for at least 20 minutes. The position of the body was standardized throughout the course of the study: the most appropriate posture (and that adopted)

was seated, with the back positioned vertically and the shins hanging down rather than positioned horizontally.

[N.B.: The above 20 minute rule was observed even if the i.v. line was already in place. If, for example, the patient was in a ward, the patient was not spoken to or allowed to move around for the 20 minutes preceding the collection of the sample for CA analysis. Similarly, any nursing procedures (e.g., physiotherapy to clear the chest) had to be stopped at least 20 minutes prior to the collection of the sample for CA analysis. (This latter guideline assumed that the patient had already been fitted with a 3-way stopcock and that the stopcock was still functional. If this was not the case, the i.v. line had to be modified accordingly and the patient allowed to rest quietly again for 20 minutes following the adjustment prior to the collection of the sample.)]

Blood was withdrawn from the subjects via the 3-way stopcocks: 2 or 3 mL were first withdrawn from the line to eliminate the i.v. fluid; then 10 mL of blood were withdrawn into a heparinized plastic syringe and the blood was immediately transfered into a cold, silicone-lined tube in an ice bucket which could be covered and protected from the light. The stopcock was simultaneously turned around so that the i.v. solution once again flushed the line clear of blood, thereby preventing clot formation. The cover was placed on the ice bucket as soon as the blood has been transferred. The subject was not engaged in conversation during this procedure. Occasionally, the use of an active i.v. line was

contraindicated; in this case, the subject was fitted with a heparin lock. It is not appropriate to collect blood for CA analysis by venipuncture.

The blood was returned to the analysis laboratory as soon as practicable and usually within 1 hour of collection. The red blood cells were centrifuged from the plasma in a darkened, refrigerated centrifuge (10 minutes at 1,200 x g). The plasma was removed from the cells and placed into cold, silicone-lined tubes in 2.5 mL aliquots. If the plasma was assayed immediately, it was maintained on ice, and in the dark, until extracted; otherwise, the sample was frozen at -70°C or colder until analysis. If a frozen sample was to be assayed for CA, it was thawed quickly by passing cold tap water over the test tube as it was shaken, only until all of the ice inside the tube had melted; the sample was then placed on ice and in the dark until it was extracted. Samples were withdrawn from the freezer only immediately before they were extracted.

2. Selection of Patients

An attempt was made to enter into the CA study all suitable patients admitted to the University of Alberta Hospitals over a six-month period. Suitable patients had a diagnosis of SAH due to rupture of a cerebral artery aneurysm. The cooperation of Drs. B.K.A. Weir and L. Disney, and the medical and nursing staff of the Emergency Department and Units 4A4 and 4A2 [Neurosurgical Intensive Care Unit and Ward] was engaged to identify all patients arriving in hospital with

the diagnosis and to alert me (D.D.) when they did. I was on call, continuously, 7 days a week, 24 hours a day, to ensure that all consecutive patients admitted to this hospital who met the inclusion criteria were included in the study [See Figure 9]. Patients were followed from the time of admission until the development and/or resolution of vasospasm; patient monitoring ranged from 1 day to 3 weeks.

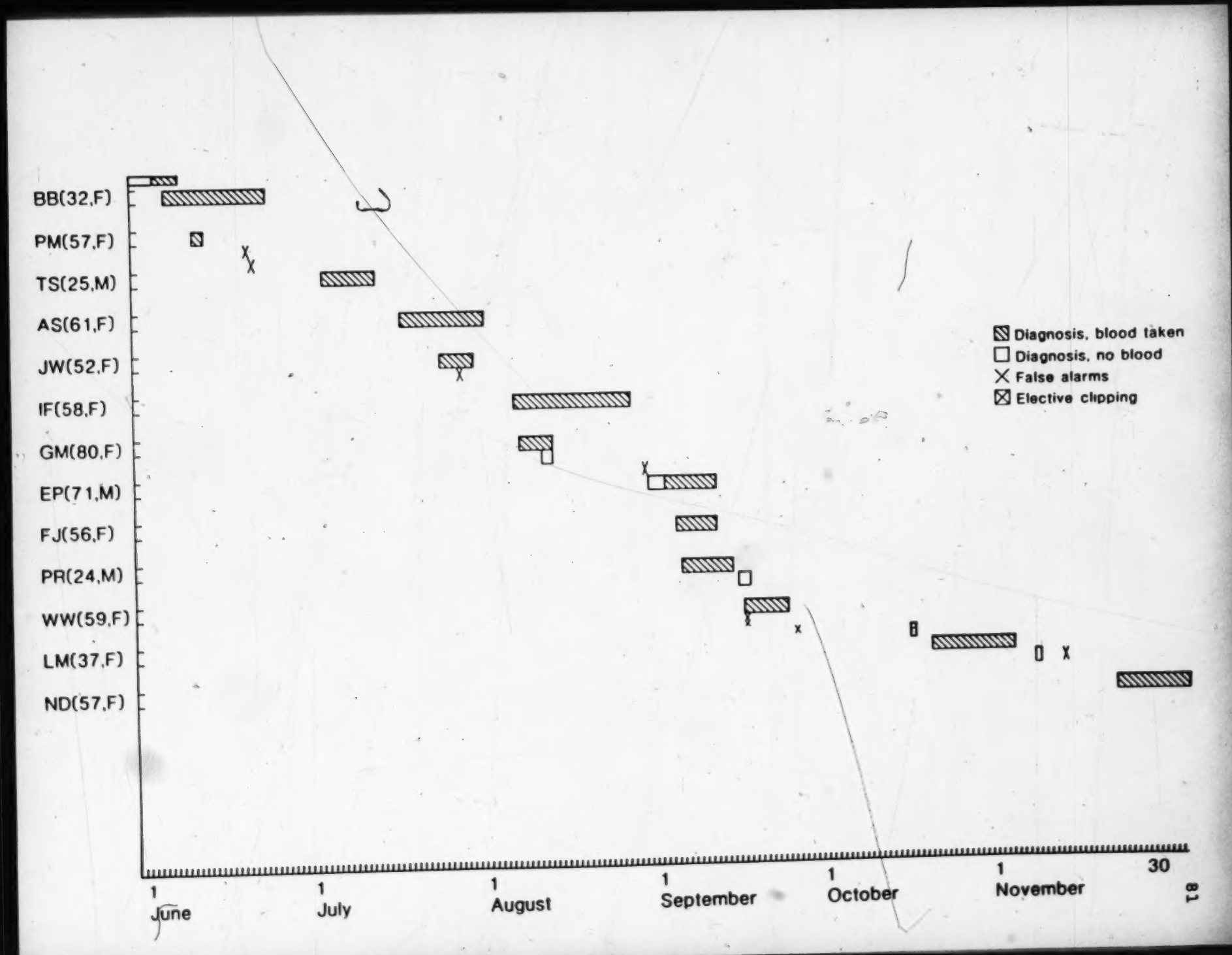
In all, I was notified of some 30 patients, 14 of whom ultimately had a confirmed diagnosis of SAH from a cerebral artery aneurysm. A number of suitable subjects for the study was missed altogether due to lack of notification. Consent (see Appendix IV) was easily obtained from all patients and/or families involved. Blood was sampled from indwelling catheters at 06:30 and 16:30 daily.

Of the 14, twelve subjects were admitted into the CA study protocol: 7 were poor-grade patients (Glasgow Coma Score, GCS, less than 10) and 5 were good-grade patients (GCS 14 or higher). Ultimately, only the plasma from the good-grade patients could be analyzed because of interferences with the CA assay from some of the drugs that had been given to the poor-grade patients.

The 5 good-grade patients comprised 3 middle-aged women (ages: 52, 57, and 59) and 2 young men (aged 24 and 25 years). They were followed for an average of 9 days.

Fortuitously, from the point of view of this study, I was contacted when the sister of one of the patients also developed a SAH due to a ruptured cerebral artery aneurysm.

Figure 9. Schedule of admission of SAH patients into the catecholamine study between June 1 and November 30, 1985.


BB(32,F)
PM(57,F)
TS(25,M)
AS(61,F)
JW(52,F)
IF(58,F)
GM(80,F)
EP(71,M)
FJ(56,F)
PR(24,M)
WW(59,F)
LM(37,F)
ND(57,F)
Diagnosis, blood taken
Diagnosis, no blood
False alarms
Elective clipping
1 June
1 July
1 August
1 September
1 October
1 November
30

This happened in Melbourne, Australia, several months after the patient here had been discharged from hospital. This situation presented a fascinating opportunity to study two siblings with a clinical problem that has a familial pattern of expression [Bannerman & Ingall, 1970]. The assistance of Dr. Murray Esler of the Baker Medical Research Institute, Melbourne, was requested in an effort to obtain at least a few blood samples from this patient so that the two sisters could be compared to one another. His cooperation extended to analyzing the collected blood samples in his own laboratory (by the REA method).

RESULTS

A. Validation of Assay Experiments

1. Polarogram

Polarograms (or "hydrodynamic voltammograms") were derived for each of the CA as described in Methods and are presented in Figure 10. [The polarograms were performed with the ECD set at 5 nA F.S.D.] The CA exhibited the classic pattern of an abrupt increase in response to applied voltage until a plateau was reached, after which time the sensitivity to applied voltage decreased again. It is apparent from the graph that NA exhibited the greatest electroactivity of the CA tested, but all were easily detectable with the HPLC-ECD system used. Based on the data obtained for the chromatography conditions selected, an operating potential of +0.72 V gave approximately maximal responses to the combination of CA of interest [See Figure 10]. Therefore, for the assays reported below, this voltage setting for the ECD was used.

All of the rest of the data reported (below) were collected with the detector operating at 1 nA F.S.D.

2. Composition of the Mobile Phase

The acceptability of phosphate as the buffering ingredient in the MP for the phenyl column had been established previously [Neill, 1984, personal communication]. The same was true for the inclusion of EDTA in the MP [Sothman, 1983, personal communication]. However, because the

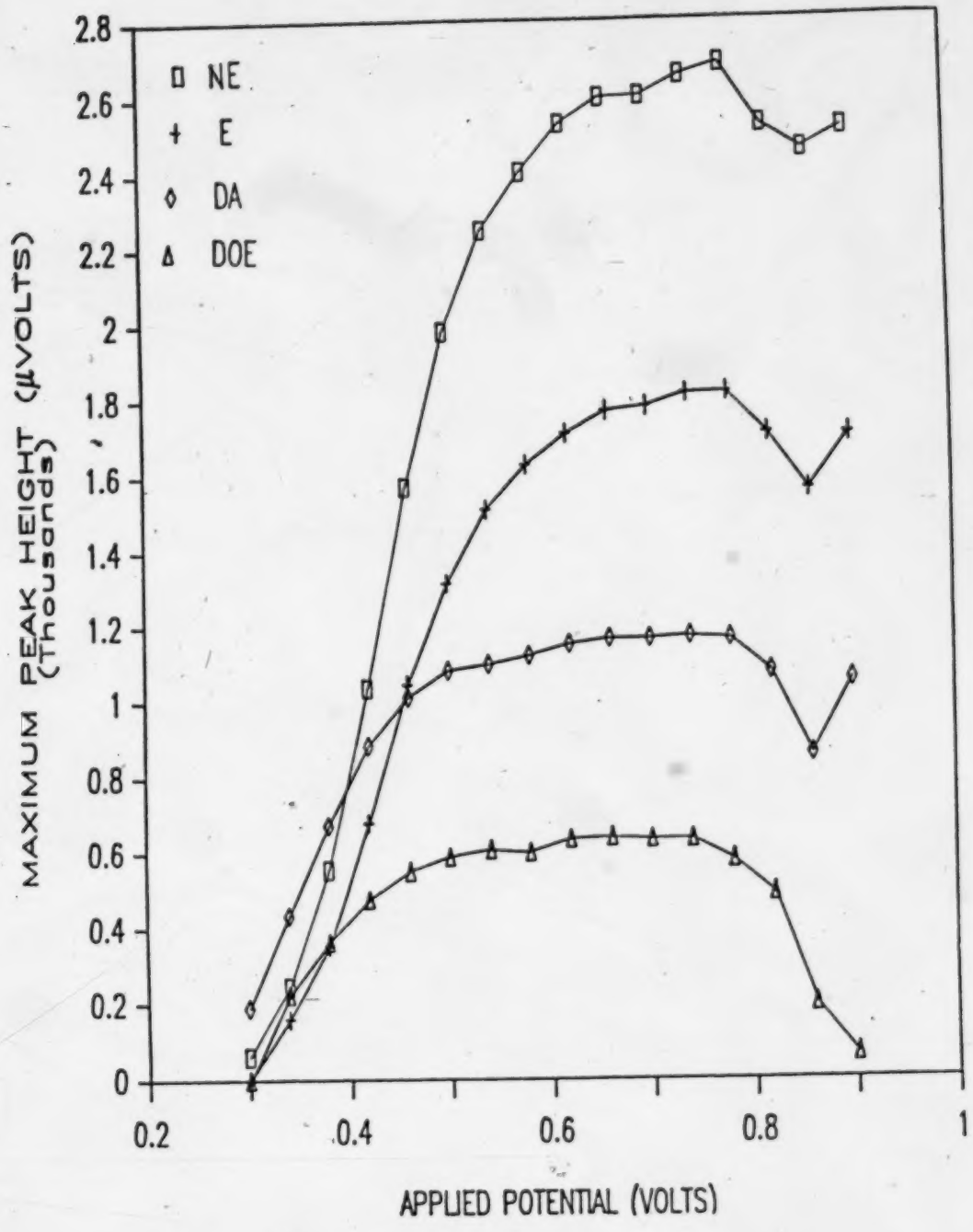


Figure 10. Polarograms for catecholamine standards injected into the HPLC-ECD system (1 ng of each catecholamine/50 μL injection volume).

basis of the interaction between the phenyl column and the CA depends on ion-pair formation, the two ingredients which modulate this pairing (namely HSA and methanol) were varied in their concentrations to determine the optimal levels necessary to achieve full resolution of the CA. The methanol was varied from 2% to 25% (v/v), and the HSA was varied from 1 to 5 mM.

A selection of chromatograms representative of the results obtained are shown in Figures 11 to 14. The chromatogram depicted in Figure 11 demonstrates the effect of a high methanol concentration (i.e., 25%) in the MP (containing 70 mM sodium phosphate, pH 4.8 at 22°C, 1 mM EDTA and 2 mM HSA). The methanol caused an alteration in the chromatogram such that the peaks for the analytes were amalgamated with the solvent front and indistinguishable from it. [In contrast, a more desirable chromatogram appeared in Figure 5].

Figure 12 demonstrates the effect of withdrawl of the methanol altogether. In this case, the composition of the MP was: 70 mM sodium phosphate, pH 4.8 at 22°C, 1 mM EDTA and 2 mM HSA. The NA peak was not well separated from the solvent front but there was no tailing of this, or the other, peaks. The baseline was choppy and the peaks were not well-formed (i.e., the peaks were not smooth in shape but rather, the signal wavered). The entire chromatogram was quite short, requiring only 8.0 minutes to complete the separation of the CA.

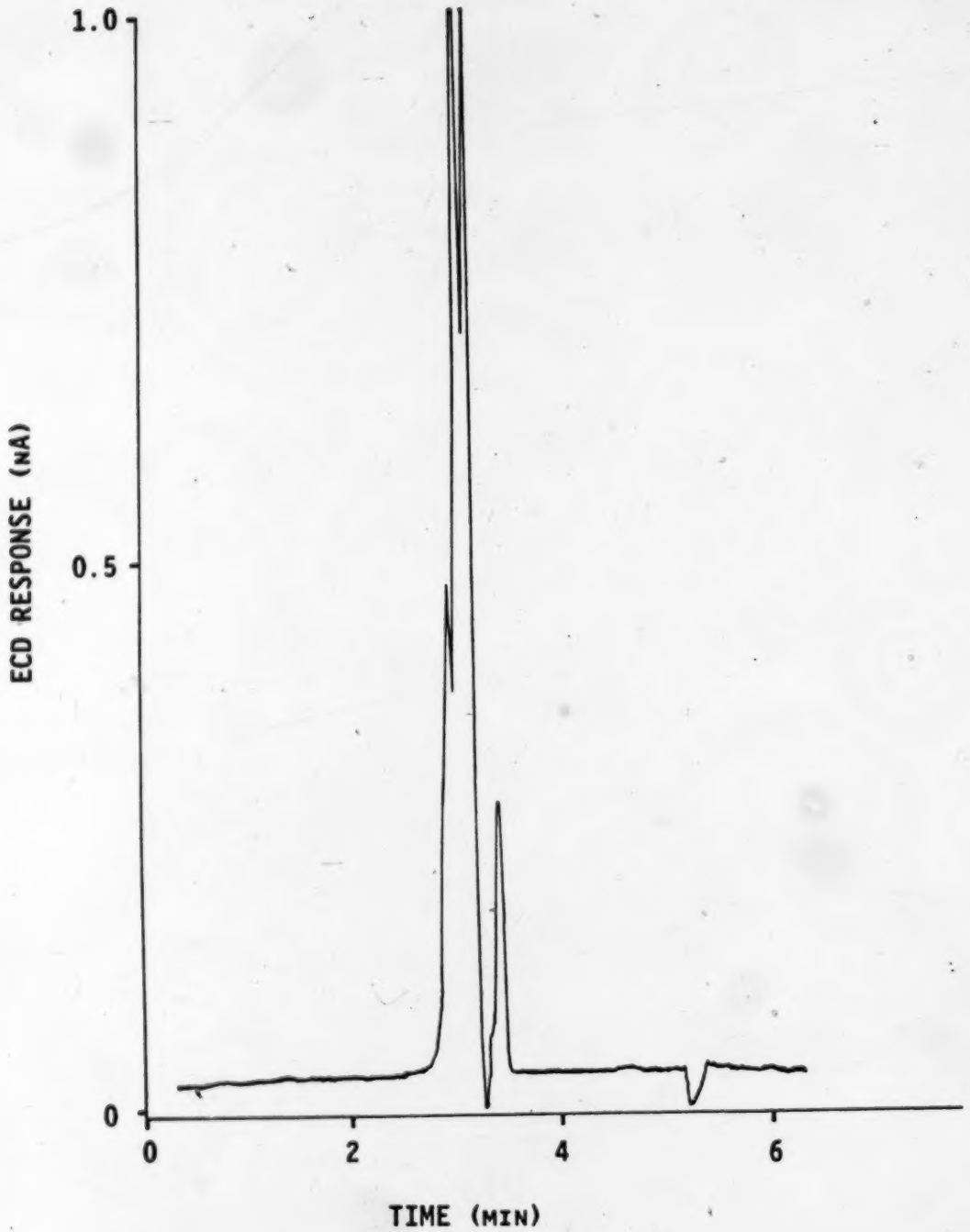


Figure 11. Chromatogram of injection of standard catecholamines with a MP containing 25% methanol and 2 mM HSA (see text for details).

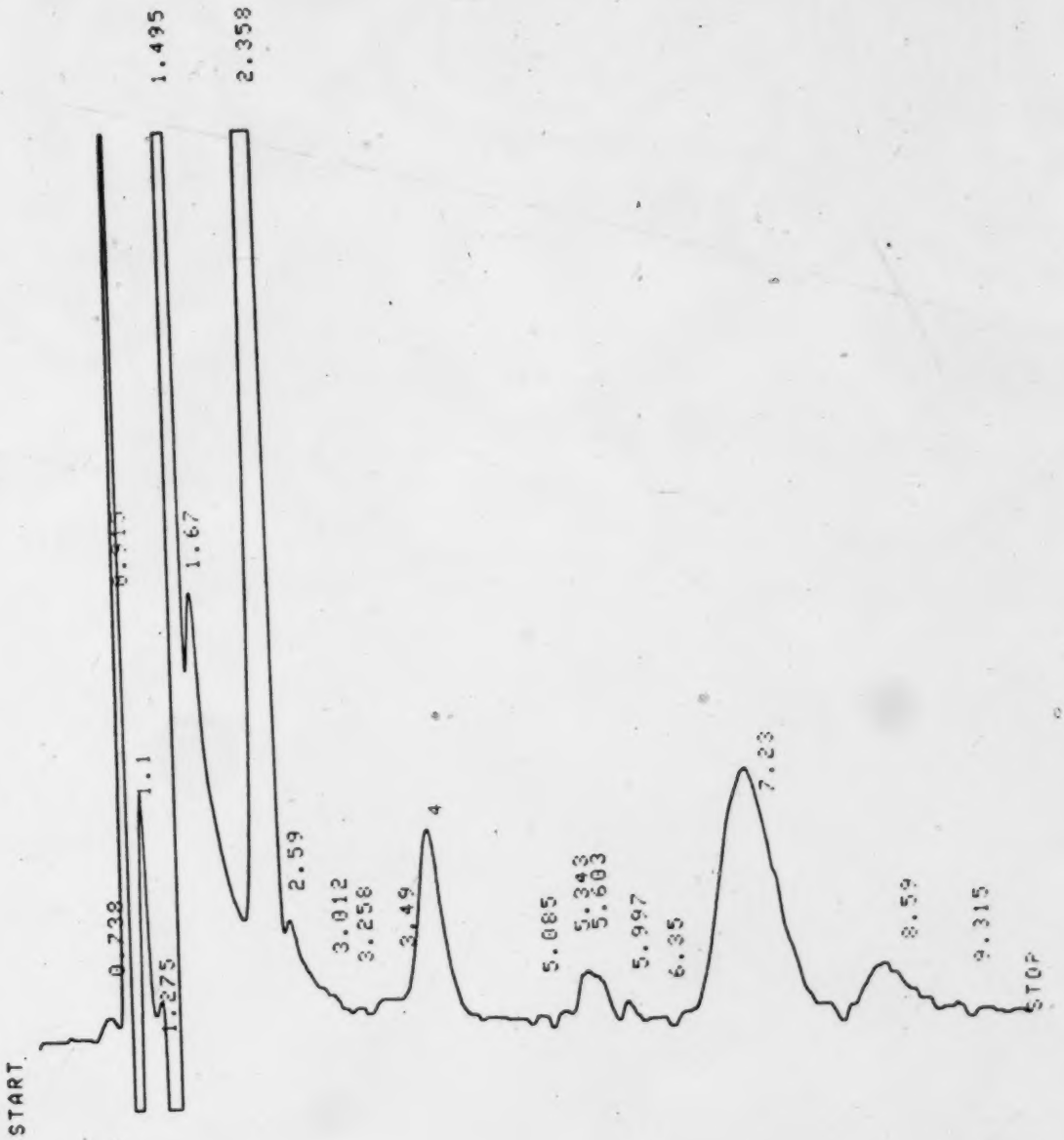


Figure 12. Chromatogram of injection of standard catecholamines with a MP containing 0% methanol and 2 mM HSA (see text for details). NA, A and DA peaks appear at 2.4, 4.0 and 7.2 min, respectively.

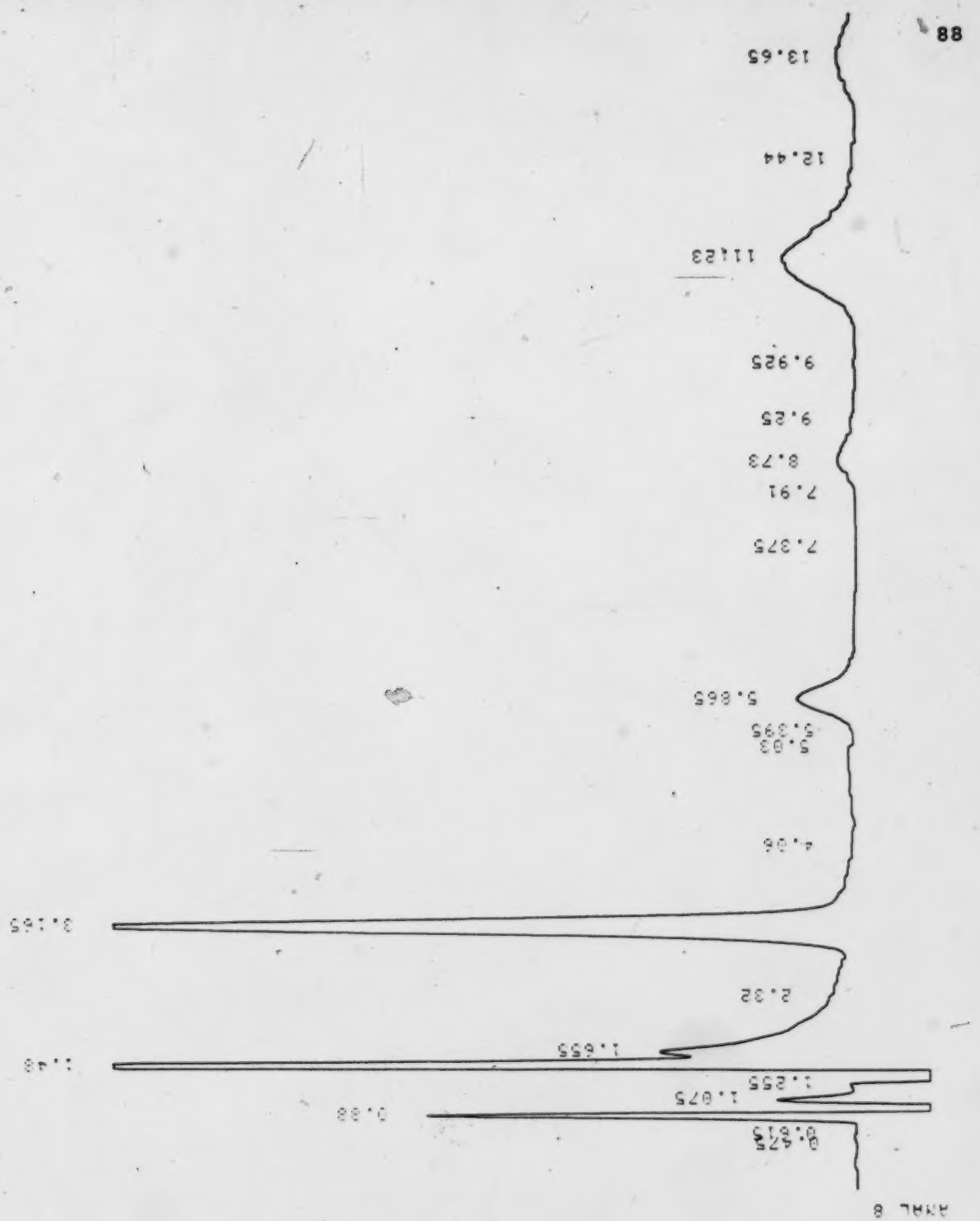


Figure 13. Chromatogram of injection of standard catecholamines with a MP containing 0% methanol and 5 mM HSA (see text for details). NA, A and DA peaks appear at 3.2, 5.9 and 11.2 min, respectively.

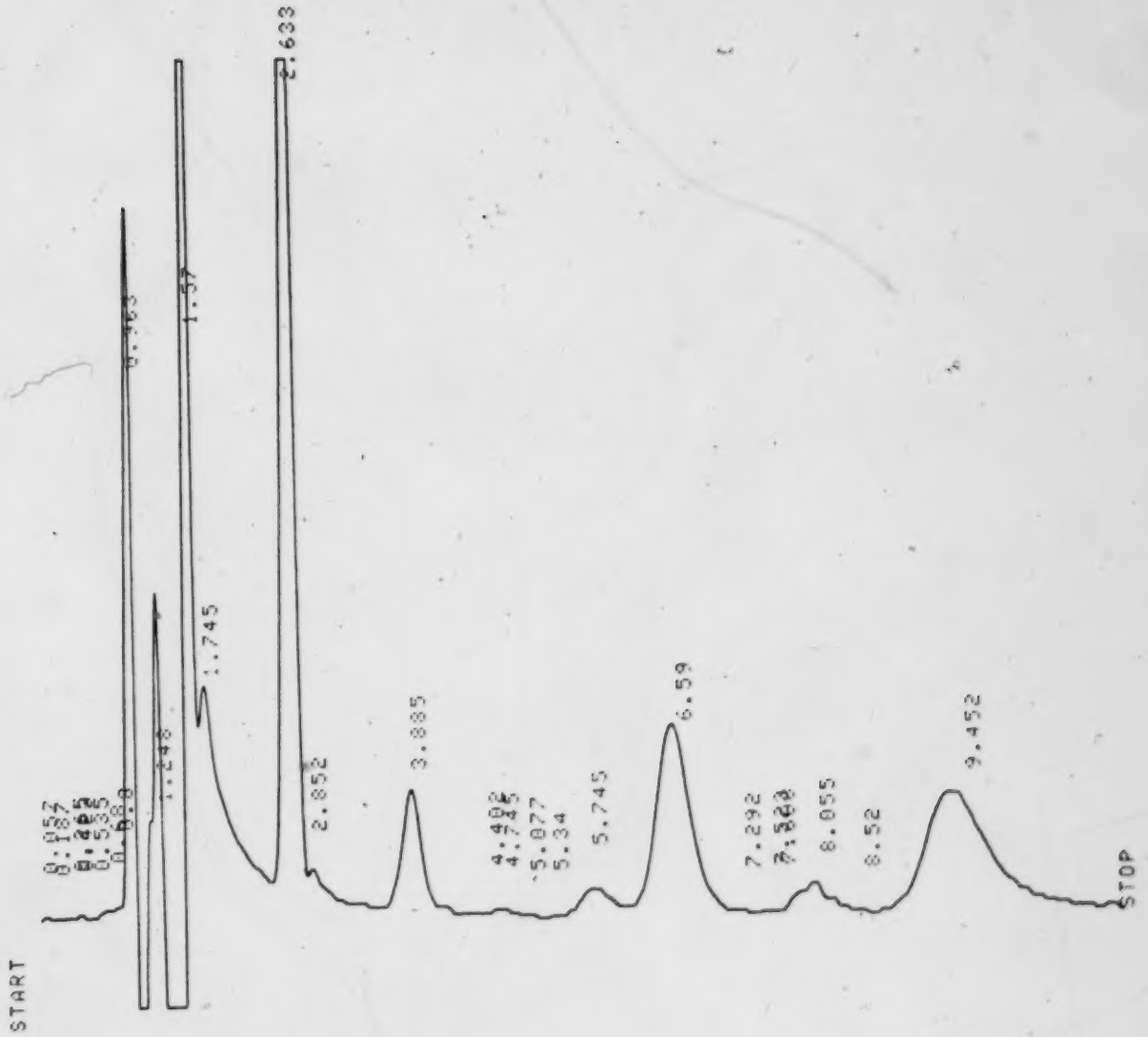


Figure 14. Chromatogram of injection of standard catecholamines with a MP containing 5% methanol and 5 mM HSA (see text for details). NA, A and DA and DOE peaks appear at 2.6, 3.9, 6.6 and 9.5 min, respectively.

Figure 13 demonstrates the effect of increasing the concentration of the HSA in the MP but without the addition of methanol. The composition of the MP in this case was: 70 mM sodium phosphate, pH 4.8 at 22°C, 1 mM EDTA and 5 mM HSA. In this instance, the chromatogram was prolonged (i.e., requiring 12 minutes to complete the desired separation) and the peaks were spread out somewhat, being both wider and more separated from each other. However, the NA peak had become well separated from the solvent front.

The chromatogram shown in Figure 14 depicts the effect of the addition of methanol (5%, v/v) to the MP (composition: 70 mM sodium phosphate, pH 4.8 at 22°C, with 1 mM EDTA and 5 mM HSA). In this case, the NA was still well resolved from the solvent front; however, peak spreading was reduced and the entire chromatogram was shorter so that the desired separation was completed within 10 minutes.

In light of this information a combination of 5% methanol (v/v) with 5 mM HSA was chosen as providing optimal resolution of the CA [as shown in Figure 14 and the Generic Chromatogram, Figure 5]. Thus, the analytical MP was selected to be: 70 mM sodium phosphate, pH 4.8 at 22°C, with 5 mM heptanesulfonate, 1 mM EDTA and 5% methanol (v/v).

Additionally, 2 "scrubbing" MPs were selected, on an empirical basis, as solutions to flush the column and ECD overnight. The purpose of these solutions was to clean the HPLC-ECD system overnight without loss of sensitivity and without the necessity for a re-equilibration period (of the

working electrode) prior to the use of the ECD on the following morning. It had been established that the boric acid extraction did not exclude large fatty acids (of the plasma) from being carried along with the CA to the final step of the extraction procedure. The presence of the fatty acids was confirmed by gas liquid chromatography (assay courtesy of Dr. D. Hadziyev, Department of Food Science of this University). These fatty acids would have been injected into the HPLC-ECD system along with the CA and would have been free to interact with the column and the surface of the WE. Any fatty acids deposited on the column would have decreased the number of sites avaliable for the chromatographic separation and fatty acids on the surface of the WE would have passivated it (i.e., causing a loss of sensitivity). Therefore, the scrubbing MPs were designed to take into consideration this source of contamination of the HPLC-ECD apparatus as well as the more common sources (e.g., other chemical impurities introduced to the flowstream and compounds leached from the apparatus).

The scrubbing MPs were: 70 mM sodium phosphate, pH 4.8 at 22°C, with 1 mM EDTA, 0.5 mM HSA and 15% methanol (v/v) for use following injections of CA standards and 70 mM sodium phosphate, pH 4.8 at 22°C, with 5 mM HSA and 15% methanol for use following injections of plasma extracts.

3. Repeatability of Injections

The variability of repeated injections of standard solutions of CA was determined by injecting (5 consecutive

times) the following concentrations (simultaneously): 2500 pg NA; 500 pg A; 1000 pg DA; and 2000 pg DOE. The coefficients of variation were found to be acceptably small: 1.8%, 1.8%, 1.3% and 0.8%, respectively (Table 2).

4. Determination of Linearity

The response of the HPLC-ECD system was found to be linear in the physiologically significant concentration range of CA. There was a straight line relationship between picograms of injected CA and the detector response to them as shown in Figures 15 to 18. The linear regression equations for the calibration curves obtained were as follows:

NA: $y = 11.59x - 320.78$; $r = 1.00$

A: $y = 7.94x - 42.29$; $r = 1.00$

DA: $y = 5.48x - 88.86$; $r = 1.00$

DOE: $y = 2.96x - 106.90$; $r = 1.00$

The retention times for each of the CA in this experiment were as follows: 2.4, 3.4, 5.4, and 7.5 minutes for NA, A, DA and DOE, respectively.

Another set of standard curves was derived from a test of the linearity of the ECD while set at the more sensitive 0.5 nA F.S.D. Triplicate injections were made of each of five concentrations of the CA, within the following ranges: 100 to 2500 pg NA; 20 to 500 pg A; 40 to 1000 pg DA; and, 80 to 2000 pg DOE. The linear regression equations obtained for these calibration curves were as follows:

NA: $y = 20.75x - 89.55$; $r = 1.00$

A: $y = 13.92x + 24.51$; $r = 1.00$

Table 2. Repeatability of standard injections of catecholamines into HPLC-ECD.

	Catecholamines Injected pg/50 µL			
	NA	A	DA	DOE
	2500	500	1000	2000
Mean Peak Height µV	23494	3144	4872	5835
Standard Deviation	431	55	62	47
Coefficient of Variation, %	1.8	1.8	1.3	0.8

N = 5
Experimental conditions: 1 nA F.S.D.

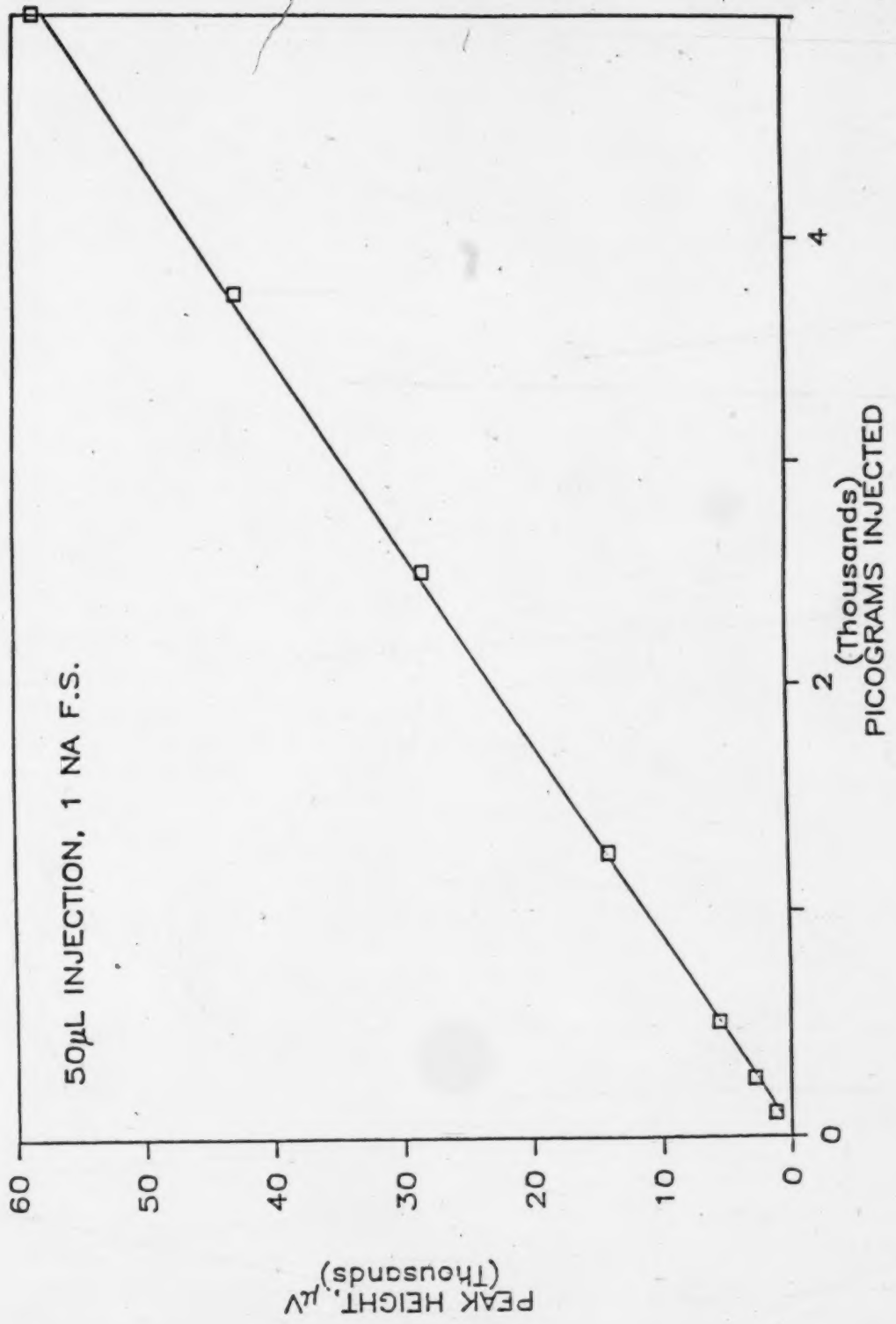


Figure 15. Standard curve for ECD response to norepinephrine.

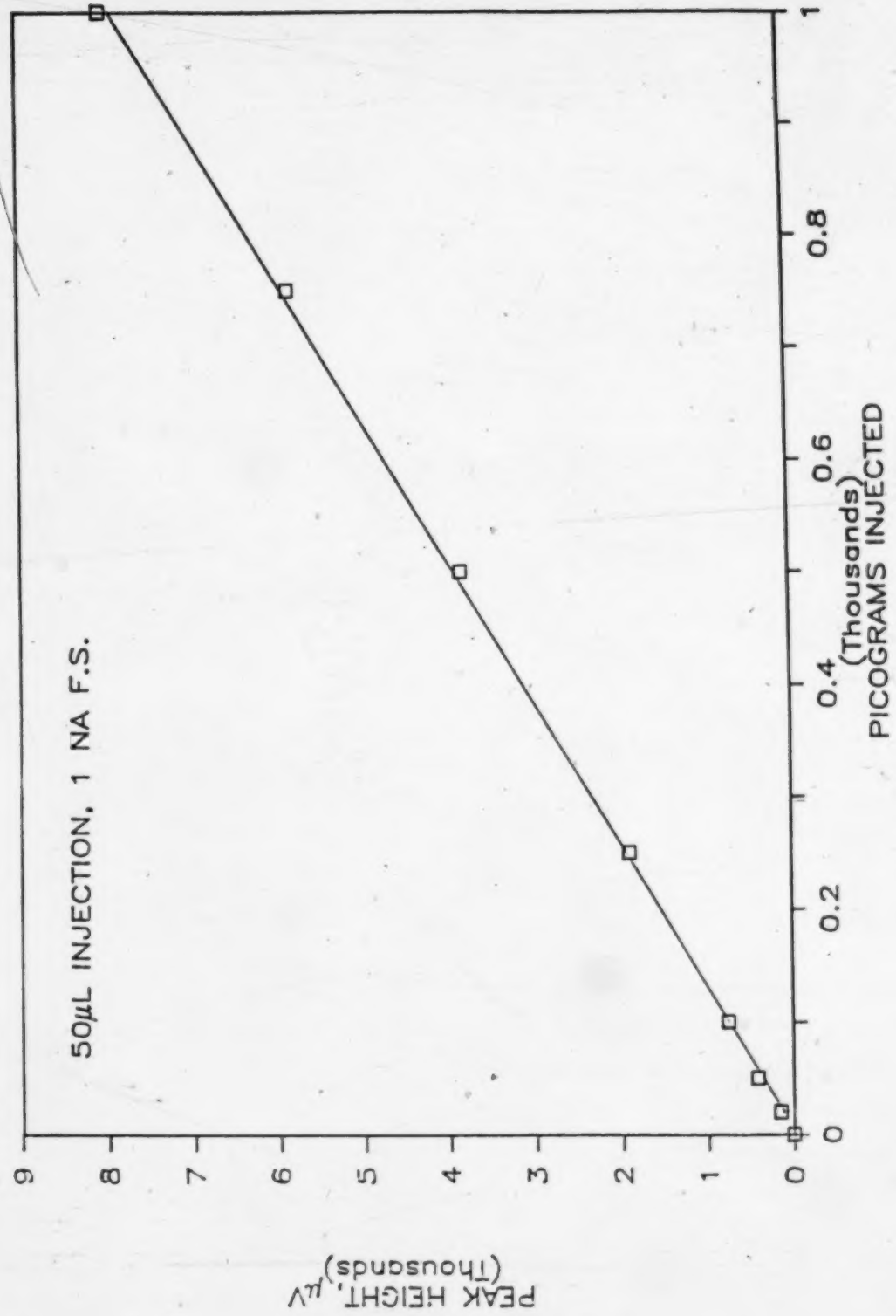


Figure 16. Standard curve for ECD response to epinephrine.

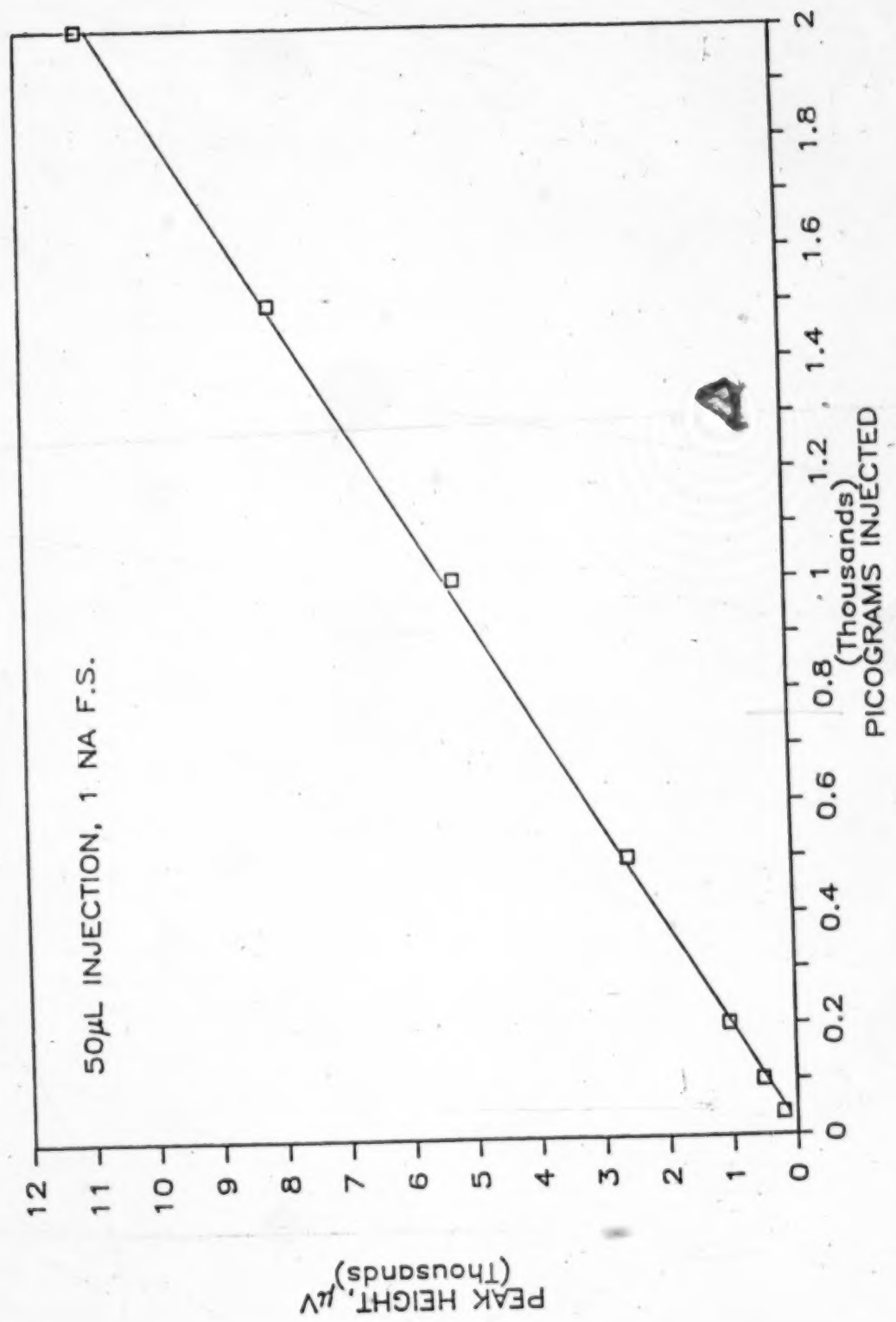


Figure 17. Standard curve for ECD response to dopamine.

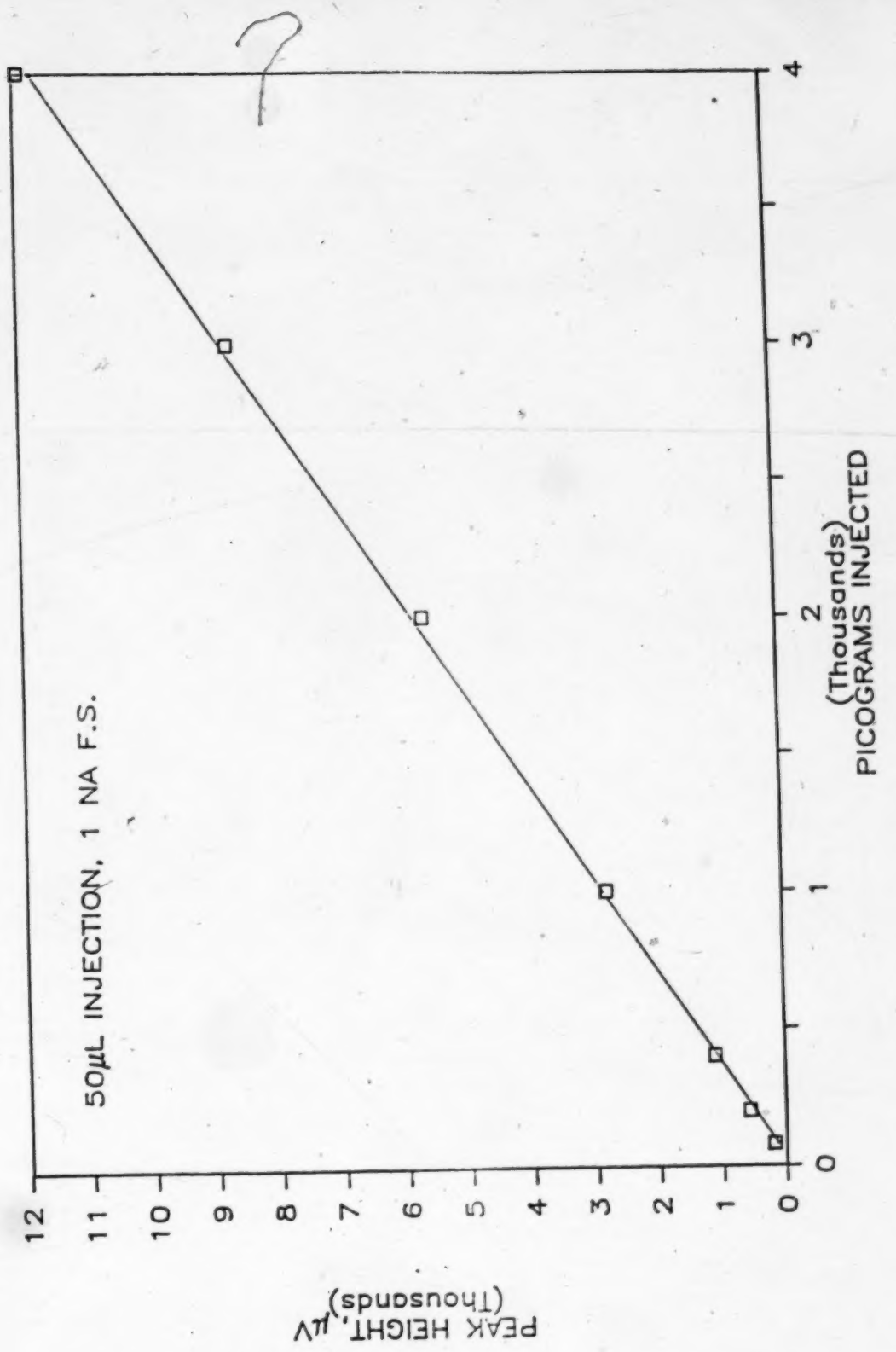


Figure 18. Standard curve for ECD response to deoxyepinephrine.

DA: $y = 9.90x - 8.25$; $r = 1.00$

DOE: $y = 5.33x - 12.96$; $r = 1.00$

The sensitivity of the ECD at 0.5 nA F.S.D. was particularly suited to the lower portion of the physiologically relevant concentration range of CA (the so-called low resting levels).

5. Determination of Per Cent Recovery

The data were obtained by adding known quantities of CA standards to CA-free plasma and then extracting and quantifying them as described in Methods. The data were calculated in terms of both absolute and relative recovery and are reported in Table 3. For the middle concentration range [Table 1], the absolute recoveries were 98% (NA), 74% (A), 85% (DA) and 78% (DOE).

However, the absolute recovery of CA from plasma varied over the physiological concentration range. Table 4 demonstrates an example of the variability of the recoveries encountered during assessments of recovery of CA from plasma in the high, medium and low (physiological) concentration ranges (as defined in Table 1). Each concentration level had a unique variability in the extent of recovery of the CA; this recovery also changed over time (in the laboratory) and appeared to be dependent on either the boric acid extraction itself or the extent of background noise in the HPLC-ECD system. Furthermore, these recoveries were also found to vary with batches of plasma; this variability was only occasionally

Table 3. Recoveries and inter-assay variabilities for extraction of CA standards from plasma.

Catecholamine	Amount Added to Plasma (pg)*	Recovery of Catecholamines from Plasma		
		Absolute Recovery (% ± S.D.)	C.V. (%)	Relative Recovery (%)**
Norepinephrine	2500	97.94 ± 1.05	1.07	124
Epinephrine	500	73.98 ± 1.63	2.20	92
Dopamine	1000	84.87 ± 3.58	4.22	107
Deoxyepinephrine	2000	77.76 ± 1.30	1.67	100

N = 3 samples extracted on each of 5 consecutive days.
C.V. = coefficient of variation.
* Amount indicated is the quantity in a 50 μL injection.
** Relative to deoxyepinephrine.

Table 4. Absolute recoveries of various concentration ranges of CA standards extracted from plasma.

Concentration Range	Per Cent Absolute Recovery of Catecholamines*			
	NA	A	DA	DOE
High	94.4 ± 2.4	80.5 ± 1.6	84.7 ± 0.5	82.1 ± 0.9
Medium	103.4 ± 0.9	83.5 ± 10.5	86.6 ± 1.3	82.9 ± 3.4
Low	130.0 ± 7.8	62.0 ± 8.8	94.5 ± 4.1	84.6 ± 3.1

Mean ± S.D.; N = 3. Experimental conditions: 1 nA F.S.D.
* Amounts of catecholamines as listed in Table 1.

attributable to the extraction of interfering substances such as drugs (data not shown).

6. Intra-Assay Variability

The coefficients of variation for the CA extracted from plasma by the boric acid extraction method, in quintuplicate, on the same day, at the medium concentration range were: 6.0% (NA), 1.8% (A), 1.4% (DA) and 1.1% (DOE) [Table 5].

7. Inter-Assay Variability

The inter-assay variability of the CA assay was assessed by extracting on 5 consecutive days, in triplicate, plasma to which had been added known quantities of CA. The coefficients of variation for NA, A, DA and DOE were: 1.1%, 2.2%, 4.2% and 1.7%, respectively [See Table 3].

8. Stability of Plasma Samples

(a) Stability of Collected Plasma Samples

The purpose of this experiment was to verify the stability of CA in plasma samples in siliconized tubes maintained on ice, and in the dark, for a short period of time after being collected (as opposed to being frozen immediately after collection). The experiment was as follows: 4 aliquots (2 mL) plasma were spiked with the medium dose of CA [Table 1], maintained on ice and assayed at the indicated time intervals [Table 6].

The data in Table 6 are presented as per cent absolute recovery. The results of the concomitant extractions of 5 aliquots of the plasma pool, spiked with the same (i.e., medium concentration level) amount of CA, are presented with

Table 5. Intra-assay variability for extraction of CA standards from plasma.

	Catecholamine Extracted pg/50 µL			
	NA	A	DA	DOE
	2500	500	1000	2000
Mean Peak Height µV	22803	2294	4107	4522
Standard Deviation	1357	42	57	51
Coefficient of Variation, %	5.95	1.8	1.4	1.1

N = 5
Experimental conditions: 1 nA F.S.D.

Table 6. Stability of catecholamines in plasma at 0°C.

Time (hours)	Per Cent Absolute Recovery of Catecholamines*			
	NA	A	DA	DOE
0 (Control)**	85.0	68.0	77.0	73.0
C.V.(%)	5.2	11.8	7.9	3.9
0	92.8	88.0	79.7	77.9
1	85.7	62.5	74.5	74.9
3	81.2	86.9	83.0	75.6

* Concentrations of CA as in Table 1, medium range; single injections.
** Control = plasma pool used for validation experiments; N = 3. C.V. = coefficient of variation.

the stability data for comparison. The results confirm that plasma samples maintained on ice, and in the dark, were stable for as long as 3 hours without significant losses of CA. No other special preservation measures were used.

(b) Stability of Frozen Plasma Samples

The longevity of the catecholamine content of plasma samples stored at -90°C was investigated. A standard quantity [i.e., the high concentration level described in Table 1] of CA was added to each of a number of paired, plasma samples which were frozen; the paired samples were thawed and assayed at intervals of 6, 9 and 20 months. These data are presented as a percentage of absolute recovery [See Table 7]. The results of this experiment indicated that the CA content of plasma frozen at -90°C does not deteriorate appreciably for almost two years.

B. Experience with the Assay

Over the course of the development of the HPLC-ECD assay described herein, a number of problems that had previously existed with this technique were identified and/or overcome. These included: (1) the stabilization of the baseline at 1 nA F.S.D., (2) the "dissection" of the chromatogram, (3) unexpected impediments to the resolution and detection of CA, and (4) the optimal polishing technique for the glassy carbon working electrode. The data obtained will be summarized in turn.

Table 7. Stability of catecholamines in frozen (-90°C) plasma.

Time (months)	Per Cent Absolute Recovery of Catecholamines*			
	NA	A	DA	DOE
0	81.0	77.0	96.0	87.0
6	85.2	83.1	85.8	85.5
9	82.8	72.9	78.0	75.7
20	91.7	74.5	100.0	92.5

* Concentrations of CA as in Table 1, high range. Single samples at each time point, injected in duplicate; mean values shown.

1. Stabilization of Baseline Noise

The aim of the protocols described in Sections A (1-4) [Methods] was to reduce the amount of baseline noise and drift at an ECD setting of 1 nA F.S.D. The use of this high sensitivity setting was necessitated by the normally very low concentrations of CA present in resting human plasma. The adoption of the methods described improved the quality of the baseline dramatically [See Figures 19 to 21]. Figure 19 demonstrates the amount of baseline noise and drift present when the HPLC-ECD apparatus was first installed. The recording was very unstable. Figure 20 demonstrates the effect of solving the problem of the chemical impurities present in the MP but not yet having conquered the electrical instabilities of operation at 1 nA F.S.D. Baseline noise and drift varied from 10 to 20% F.S.D. Figure 21 demonstrates the baseline that was achieved when all of the measures described in sections A (4) in Methods were adopted. In this case, the baseline did not drift, there was no electrical interference present, and the noise was less than 2% F.S.D.

2. Dissection of the Chromatogram

The chromatograms referred to in the following discussions were all generated by the Shimadzu C-R3A integrator. The ATTENTUATION of the Shimadzu was set at 3; this corresponded to 0.8 nA F.S.D. on the paper (but the ECD was still set at 1 nA F.S.D.). Peak height is reported in μV.

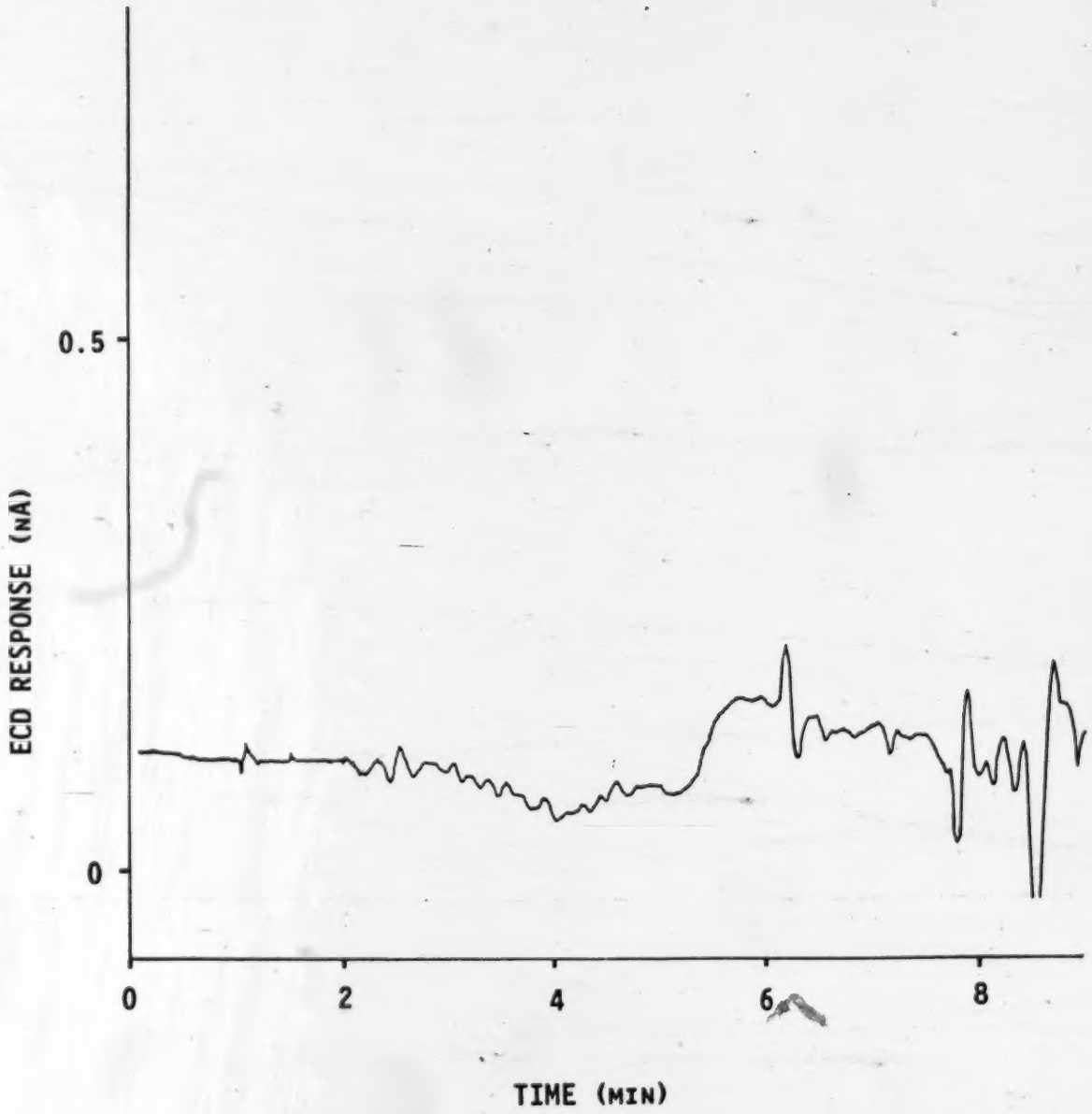


Figure 19. Baseline signal from ECD prior to development of mobile phase and optimal operating conditions of the HPLC.

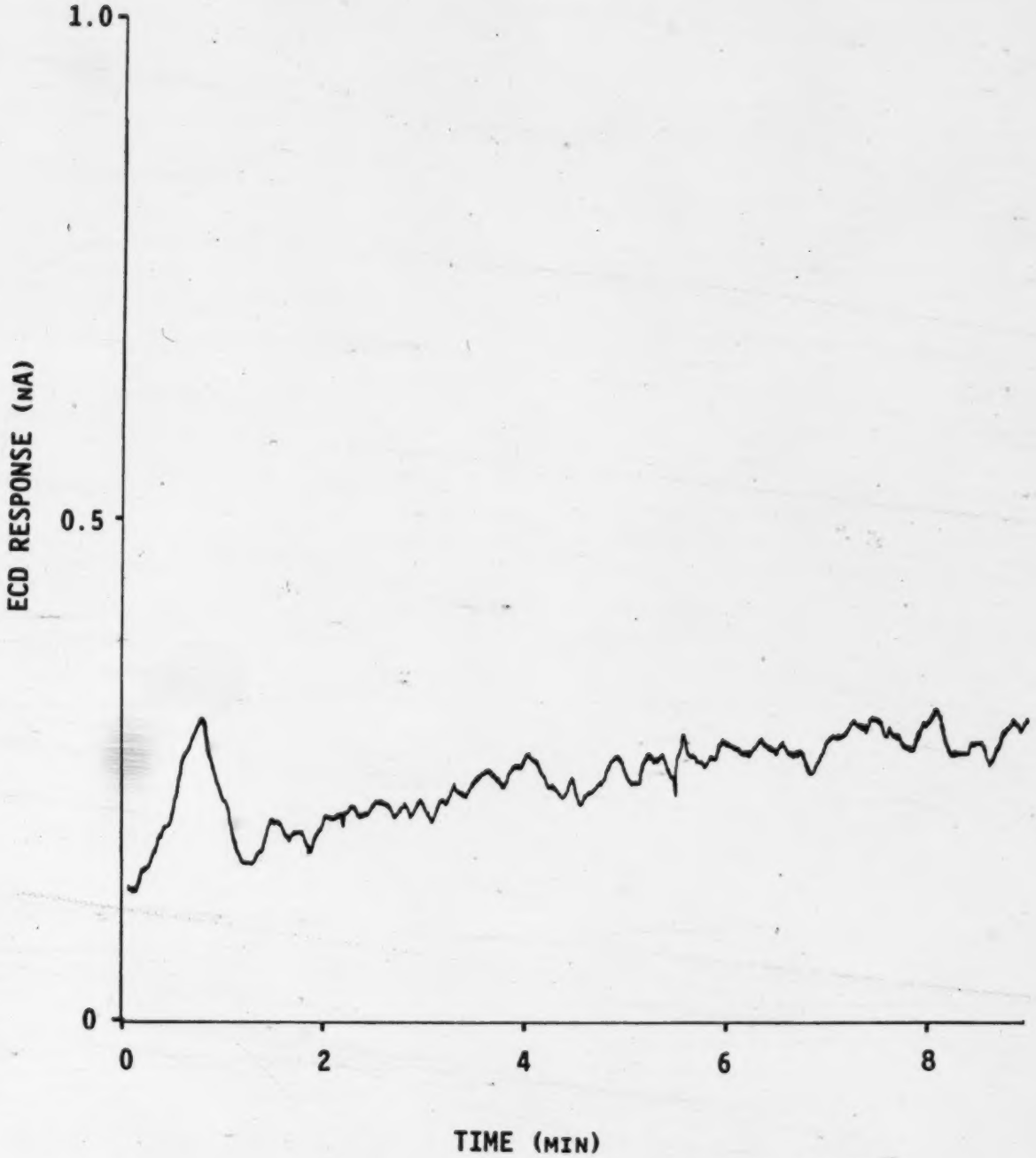


Figure 20. Baseline signal from ECD during development of optimal operating conditions of the HPLC. (Peak at the left is DOE.)

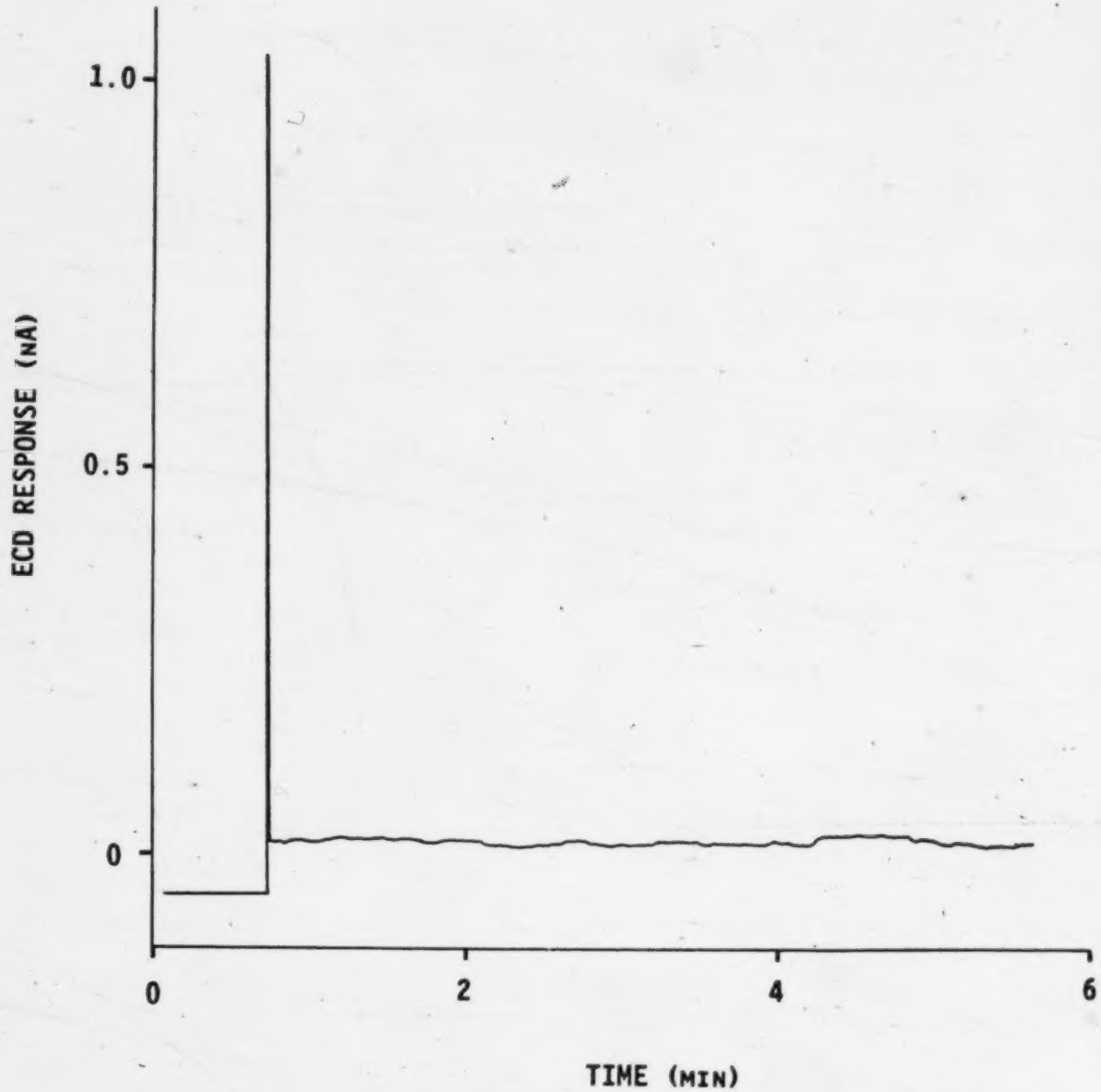


Figure 21. Baseline signal from ECD at optimal operating conditions of the HPLC.

The integrator does provide a calculation of the concentration of the analyte in the peak on the basis of the height of the internal standard peak ("CONC" on the report of the chromatogram, presented below the tracing) but this information was not utilized. Concentrations of CA in the original plasma sample (expressed as pg of CA/mL plasma) were calculated individually according to the following formula:

$$\left[\frac{\frac{\mu V \text{ unknown CA}}{\mu V \text{ I.S. peak}}}{\frac{\text{slope of CA}}{\text{slope of I.S.}}}\right] \times \text{amount of I.S.} \times \text{R.F.} \times \text{V.C.}$$

where: I.S. = Internal Standard

Amount of I.S. = Amount of I.S. (pg) in I.S. peak (calculated daily based on injection of standard solution)

R.F. = Recovery Factor (i.e., compensation for absolute recovery of CA during extraction from plasma)

V.C. = Volume Correction (i.e., compensation for volume of plasma extracted and injected)

slope = Slope of linear regression equation (i.e., from the standard curves)

All injections into the HPLC-ECD system were made with the sample to be analyzed dissolved in a carrier solution of 80 mM acetic acid. Most often, the actual injection volume was 50 µL. Figure 22 demonstrates that component of the chromatogram generated by this carrier solution. The acetic acid was primarily responsible for the width of the solvent front; the peak present at 4.83 minutes was a constituent of the water, not the acetic acid (chromatogram for water alone not shown).

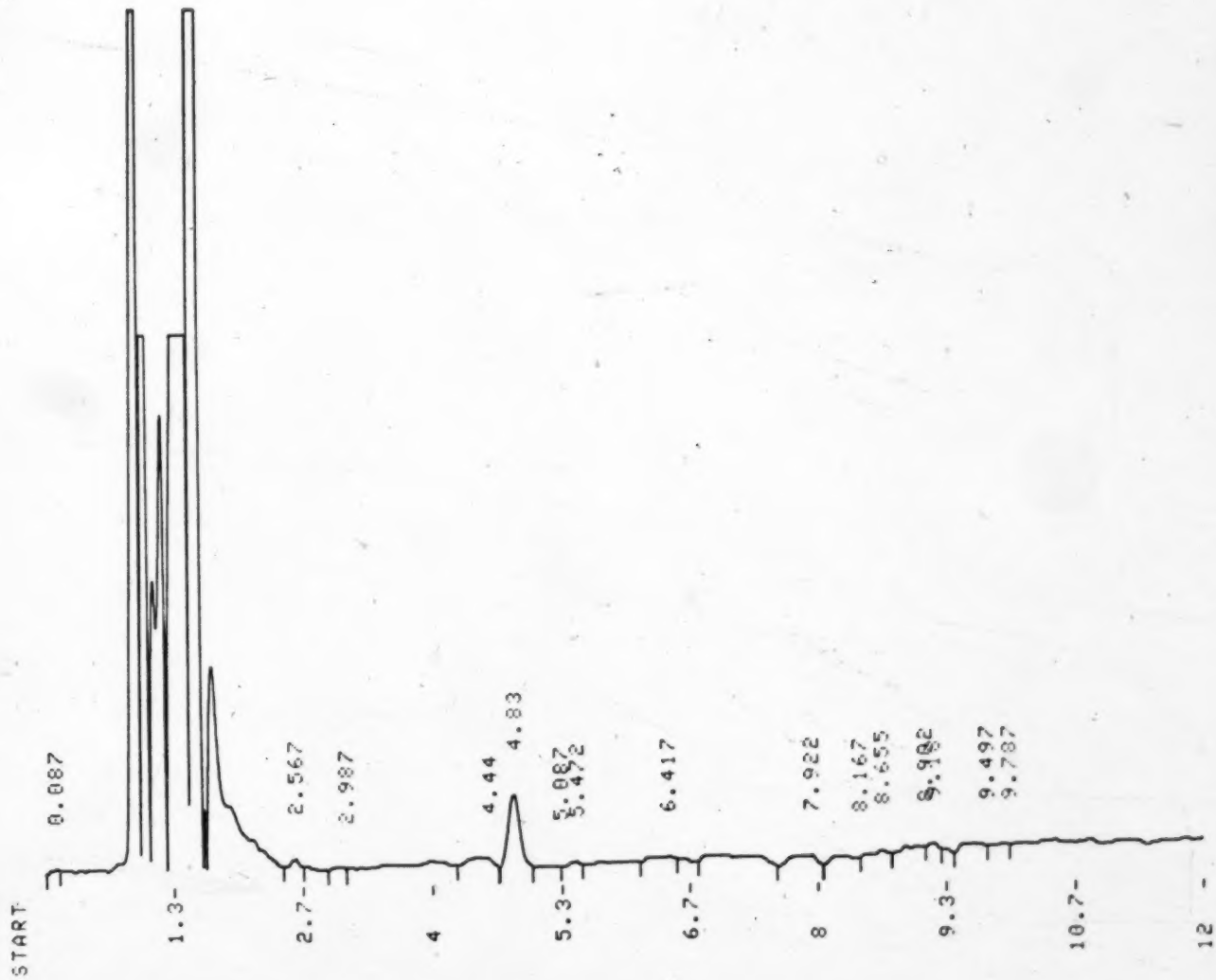


Figure 22. Chromatogram for injection of carrier solution for catecholamine standards and plasma extracts (50 μL of 80 mM acetic acid).

Figure 23 demonstrates the chromatographic resolution of the CA that was achieved by the conditions described herein. This is a chromatogram of a 50 μL injection of a solution of standard CA containing 500 pg NA, 100 pg A, 200 pg DA and 400 pg DOE (i.e., picograms injected onto the column). The retention times of the 4 CA were: 2.5, 3.5, 5.6 and 7.7 minutes for NA, A, DA and DOE, respectively.

A typical chromatogram of an extracted, resting human plasma sample is shown in Figure 24. The extent of separation of the NA from the solvent front was highly variable and strongly influenced by the presence of drugs (or drug metabolites) in the plasma sample. One of the most frequently encountered drugs which affected this portion of the chromatogram (solvent front) was ephedrine [See Figure 25]. This is a commonly administered sympathomimetic with a basic molecular structure very similar to that of the CA.

3. Unexpected Impediments to the Resolution and Detection of CA

Even if one has succeeded in once overcoming the chemical and mechanical difficulties associated with extracting, separating and detecting the sample to be analyzed, there remain other limitations to obtaining clear data from the ensuing chromatograms. These other limitations encompass things which are outside the control of the CA investigator yet which are capable of generating serious difficulties for the analytical laboratory. Examples of such impediments

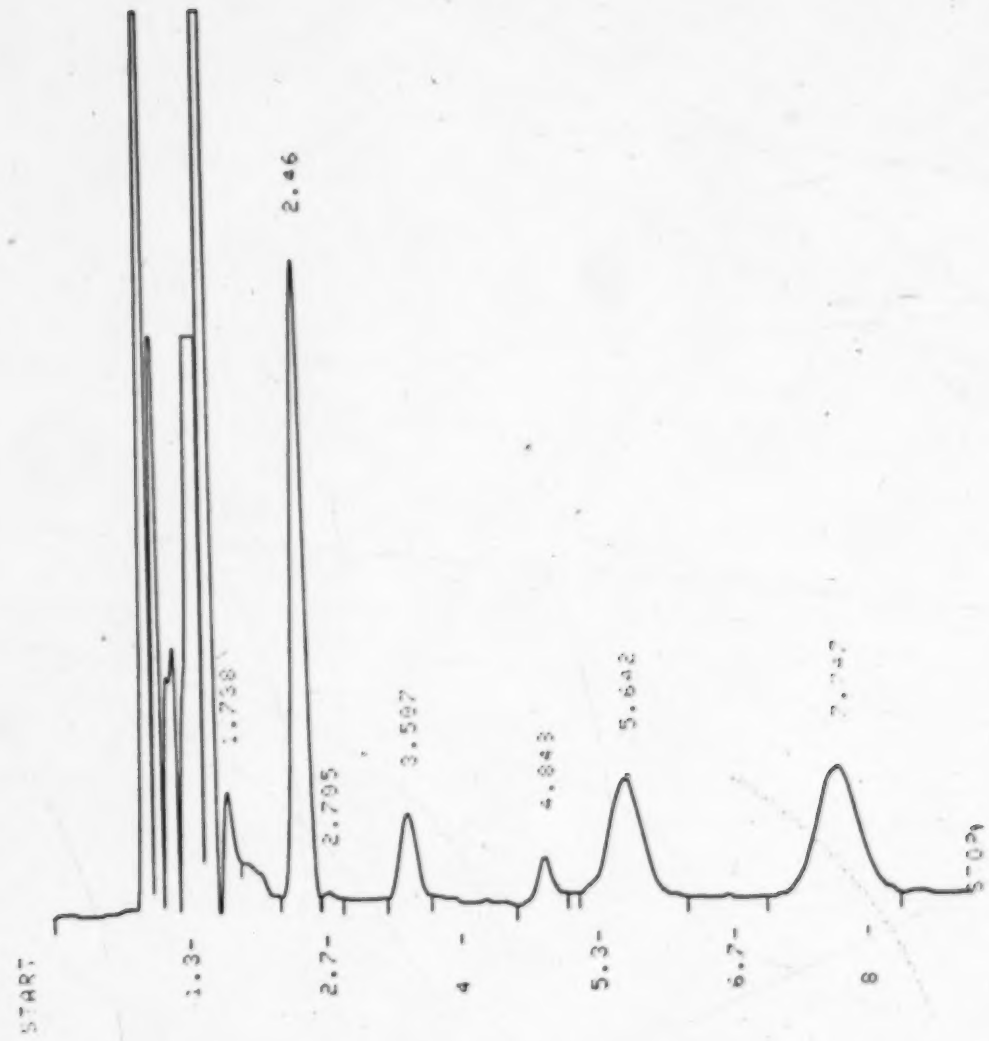
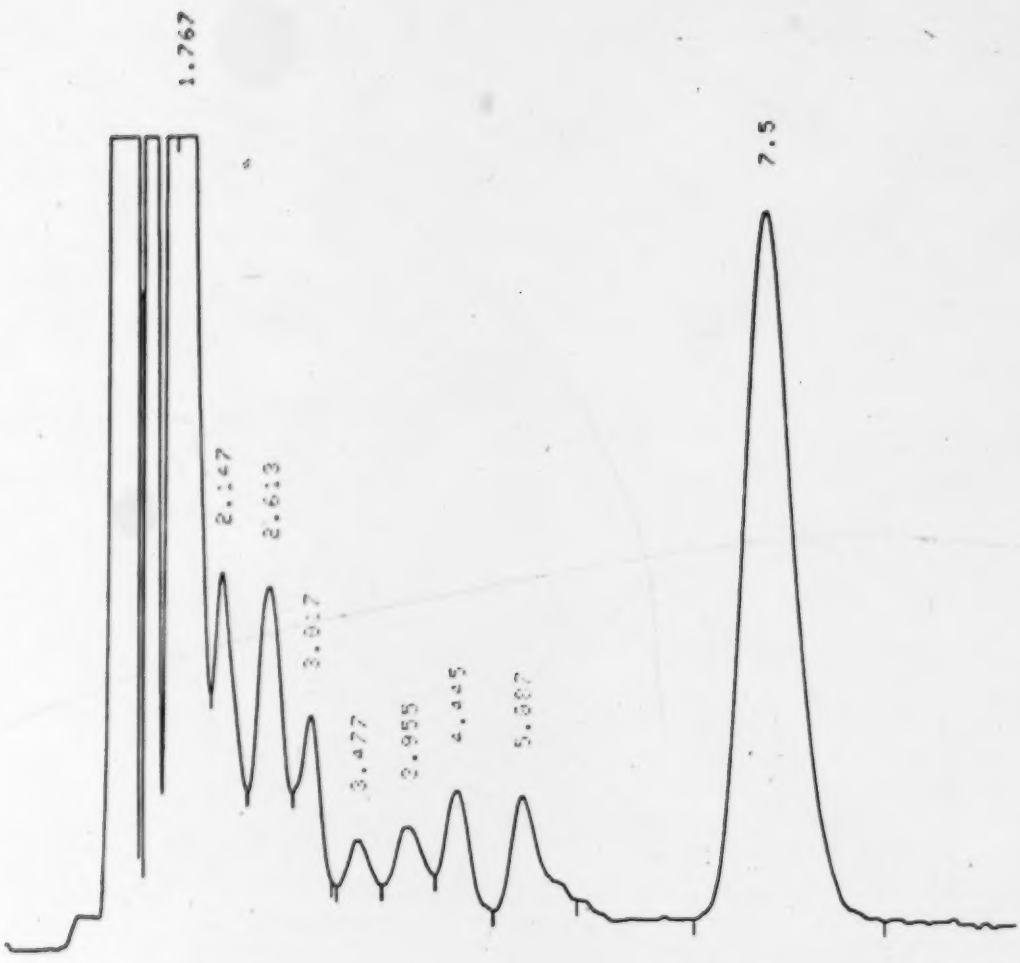
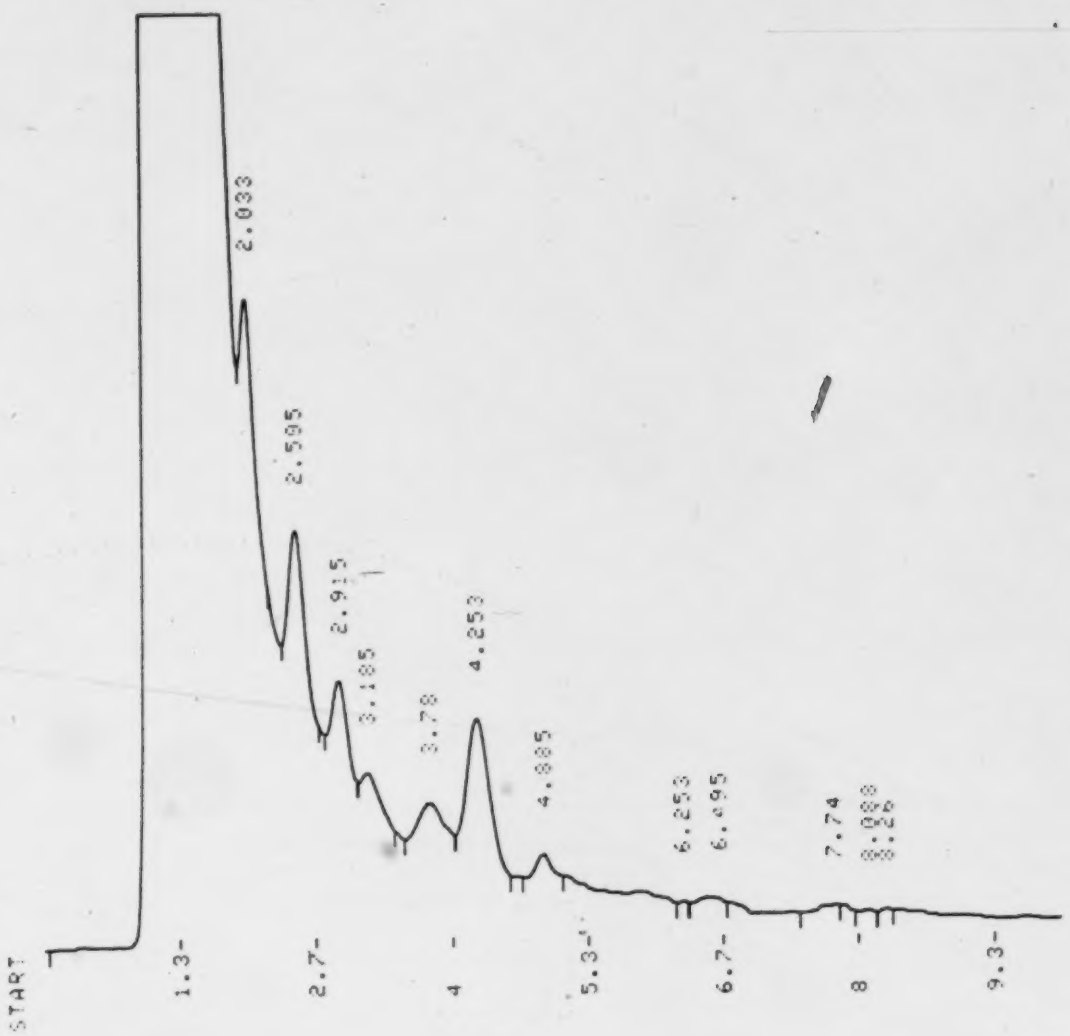


Figure 23. Chromatogram of standard catecholamines (50 µL, 1 nA F.S.D., 500 pg of NA, 100 pg of A, 200 pg of DA and 400 pg of DOE). NA, A, DA and DOE peaks appear at 2.5, 3.5, 5.6 and 7.7 min, respectively.



PKNO	TIME	HIGHT	MK	IDNO	CONC	NAME
3	2.613	1818		2	259.5042	NOREPI
5	3.477	459		3	82.9187	ADREN
9	7.5	6244		1		EPININE
	TOTAL	8471			342.4229	

Figure 24. Chromatogram of an extract of human plasma.



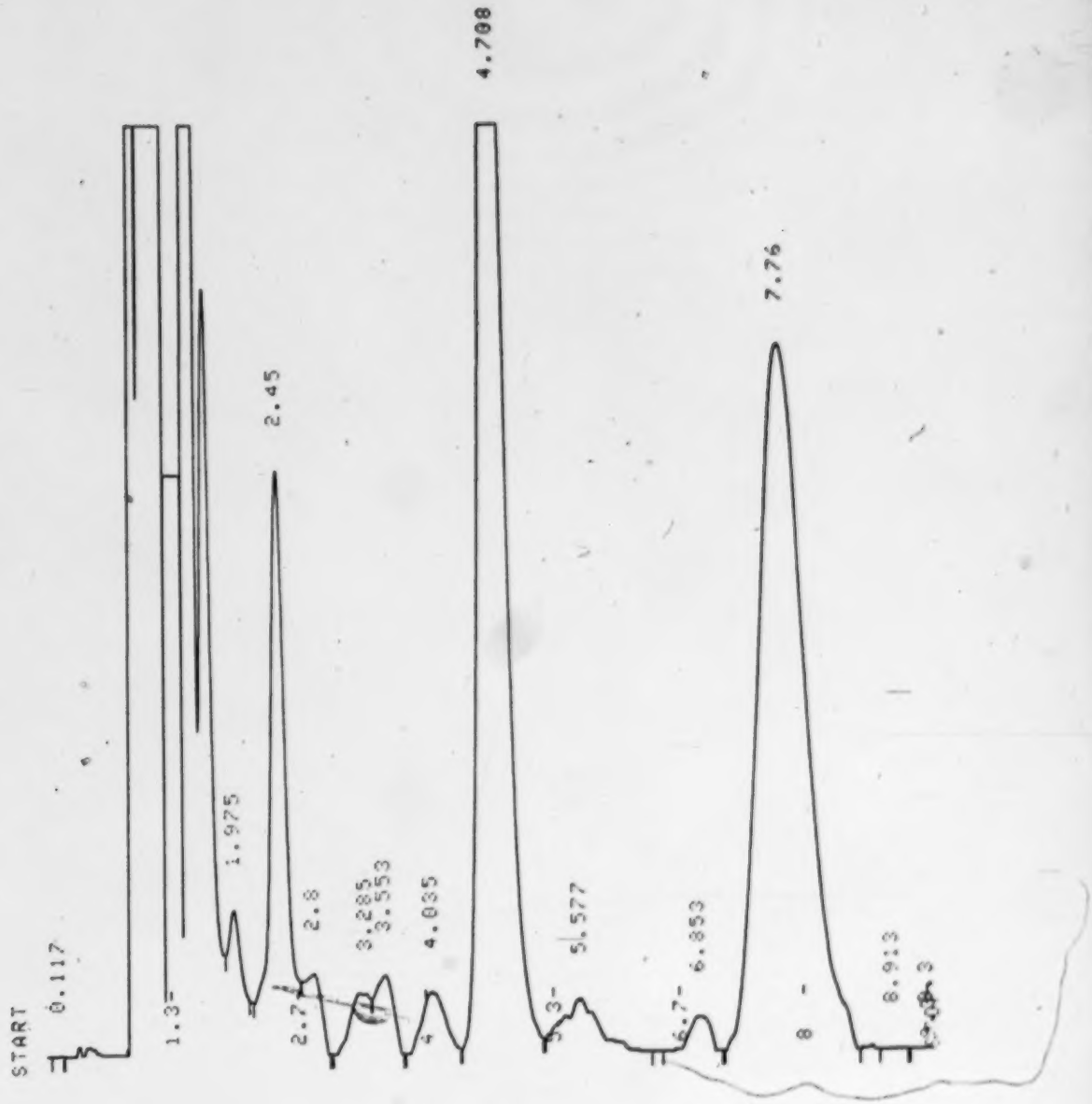
PKNO	TIME	-IGHT	IDNO	CONC	NAME
2	2.505	2602	2	14963.9736	NOREPI
9	7.74	72	:		EPININE
10	8.088	62	:	1733.6003	EPININE
	TOTAL	2736		16697.5722	

Figure 25. Chromatogram of an extract of human plasma known to contain an anti-histamine.

include drugs/substances ingested by the subjects under study and treatment of replacement parts for the HPLC-ECD apparatus by the manufacturers of the parts. This latter issue encompasses the extent of the quality control exerted by the manufacturer and all events which take place between the time the item is manufactured and its delivery to the CA laboratory (e.g., how it is packed in the shipping crate).

The extent of the interference of drugs (e.g., administered to hospitalized SAH patients) with the HPLC-ECD CA assay was not known prior to the start of this study. There are a number of potential problems associated with the presence of drugs in the plasma sample. Excluding the possible effects of drugs on endogenous levels of plasma CA, the drugs may be extracted from the plasma along with the CA and proceed to interact with the HPLC-ECD apparatus, and finally, alter the chromatogram. There are a number of possible interaction sites along this cascade of events and include deposition of the drugs on the column (thereby lowering the number of chromatographic interaction sites) and deposition of the drugs on the surface of the WE (thereby passivating it and lowering its sensitivity). Possible effects on the chromatogram could range from no interference with the separation of the CA (in spite of the presence of drugs in the sample) to a complete obliteration of discernable CA peaks because of interfering peaks from the drugs.

The former case is illustrated in Figure 26. The peaks on the chromatogram assumed to be due to the presence of drugs



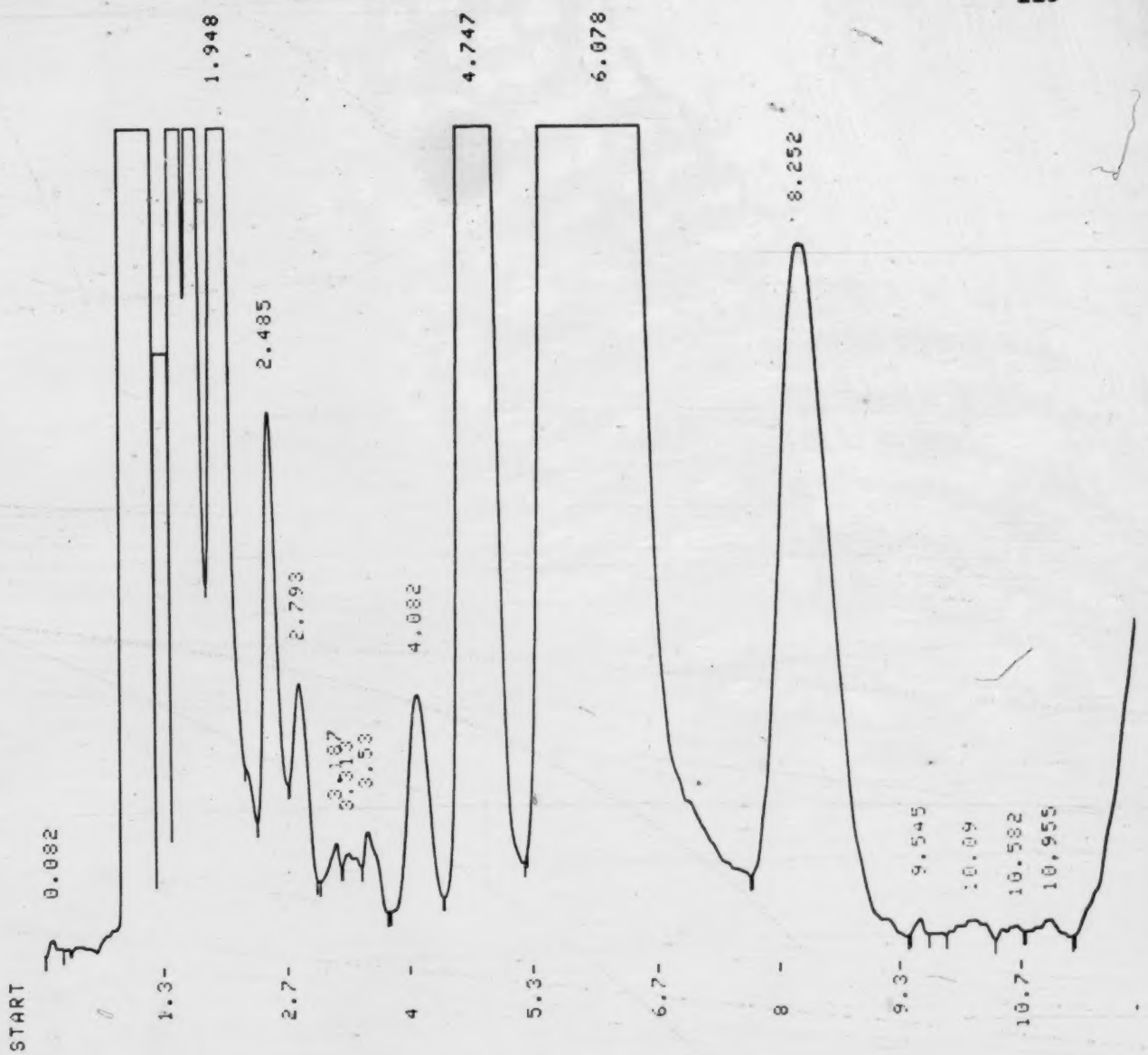
PKNO	TIME	-IGHT	MK	IDNO	CONC	NAME
3	2.45	4885		2	582.7805	
5	3.285	251		3	36.6594	
9	5.577	416		4	125.1533	
11	7.76	6722		1		
	TOTAL	12275			744.5932	

Figure 26. Chromatogram of an extract of human plasma known to contain various drugs and their metabolites.

in this plasma sample (i.e., at 2.0, 4.1, and 6.9 minutes) did not interfere with the resolution of the CA (at 2.5, 3.3, and 7.8 minutes, respectively, for NA, A and DOE). [The following drugs had been administered to this patient in the twelve hours preceeding the blood sample: Ventolin, Dilantin, codeine, Valium, Tempra, Gentamycin, Vancomycin, Penicillin, and heparin.]

Another problem that was encountered in association with the group of SAH patients was the effect of a DA infusion on both the endogenous levels of CA and on the final chromatogram. This is illustrated in Figure 27. In this case, the presence of large amounts of DA (and breakdown products of DA) in the plasma created numerous other peaks on the chromatogram and interfered with the resolution of the A peak. This kind of interference rendered the accurate quantification of (in this case) A impossible. [It is worthy of note that the patient from whom this blood sample was taken, and from which the chromatogram in Figure 27 was generated, had been administered the following medication in the twelve hours preceeding the collection of the blood sample: Ventolin, Dilantin, heparin, Gentamycin, Vancomycin, albumin, Lasix, heparin, Tempra, codeine, dopamine, Penicillin, Pitressin and KCl solution.]

Another identified source of impediments to the resolution and detection of CA is damage to the chromatographic bed due to mechanical shocks suffered by the column (either in-transit from the manufacturing plant or else



PKNO	TIME	HIGHT	IK	IDNO	CONC	NAME
2	2.485	3816		2	482.4389	
5	3.53	454		3	70.22	
8	6.078	134051		4	42708.8789	
9	8.252	6343		1		
	TOTAL	144665			43261.5351	

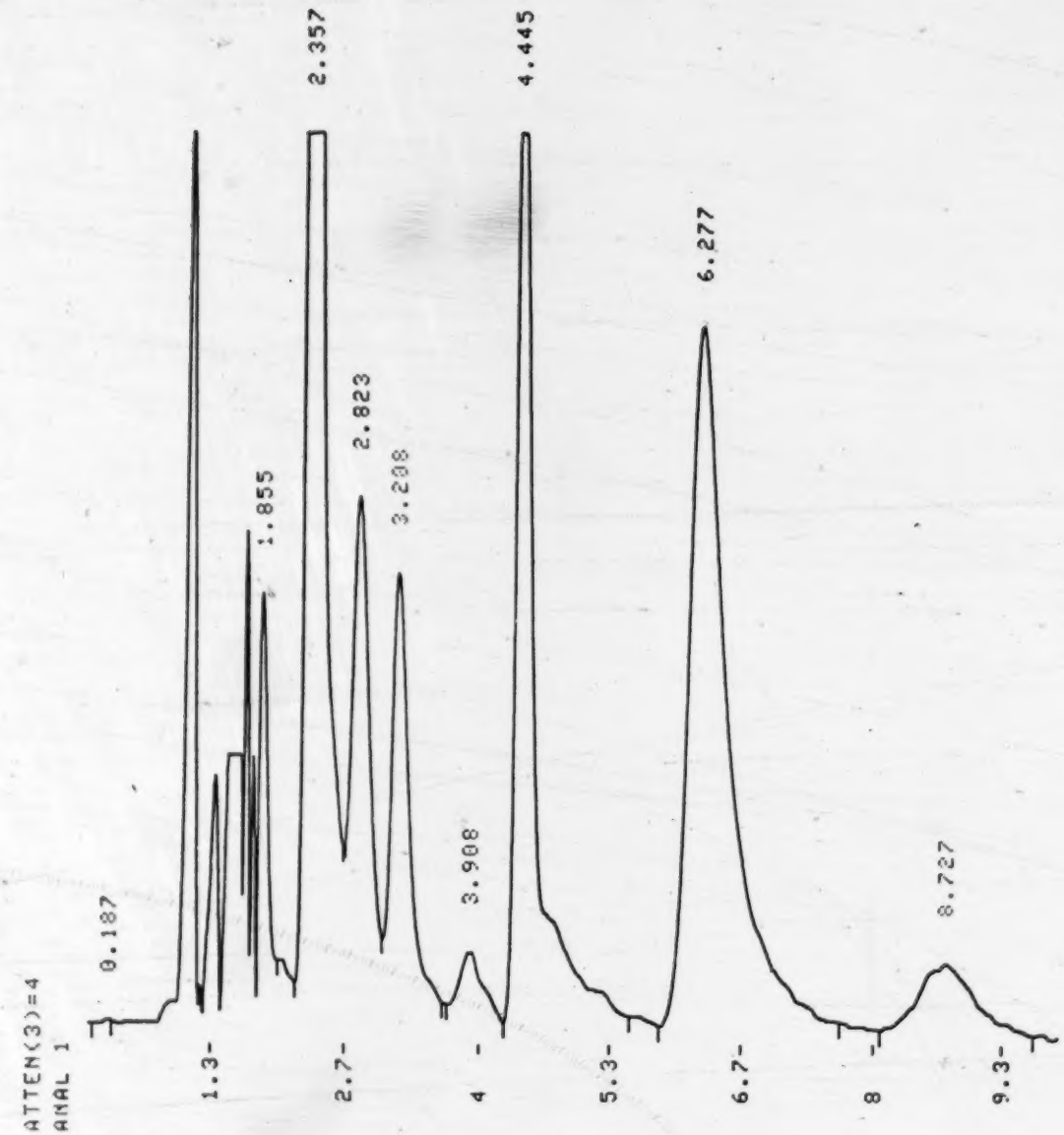
Figure 27. Chromatogram of an extract of human plasma known to contain various drugs, their metabolites and dopamine.

in the CA laboratory during repairs to the apparatus). In this case, the sensitivity of the ECD cell would be unaffected but the chromatographic resolution of the CA would be diminished.

An example of the kind of chromatogram generated by this problem is shown in Figure 28. This tracing resulted from the injection of 50 μL of a standard CA solution containing 5000 pg NA, 1000 pg A, 2000 pg DA and 4000 pg DOE. It is evident from the peak heights that the sensitivity of the detector was high (compare μV generated by these peaks ("HIGHT") with peak heights, in μV, of the CA on the standard curves, Figures 15 to 18). However, the peaks on this chromatogram were split (e.g., NA at 2.4 and 2.8 min) and had a sharp rise and scree-slope shoulders, rather than a Gaussian shape. The elution time for the chromatogram was also shortened compared with that usually seen under the same chromatographic conditions. These results indicated that the column was not interacting properly with the analytes. Three columns, when received from the manufacturer, displayed these problems and the manufacturer confirmed that other users had had the same experience with the same lots of columns.

4. Polishing of the Glassy Carbon Electrode

Many of the problems described above ultimately lead to passivation of the surface of the glassy carbon electrode and diminution of its sensitivity. Some means of restoration of function of this electrode must be utilized on a regular basis to re-establish its sensitivity. This is particularly



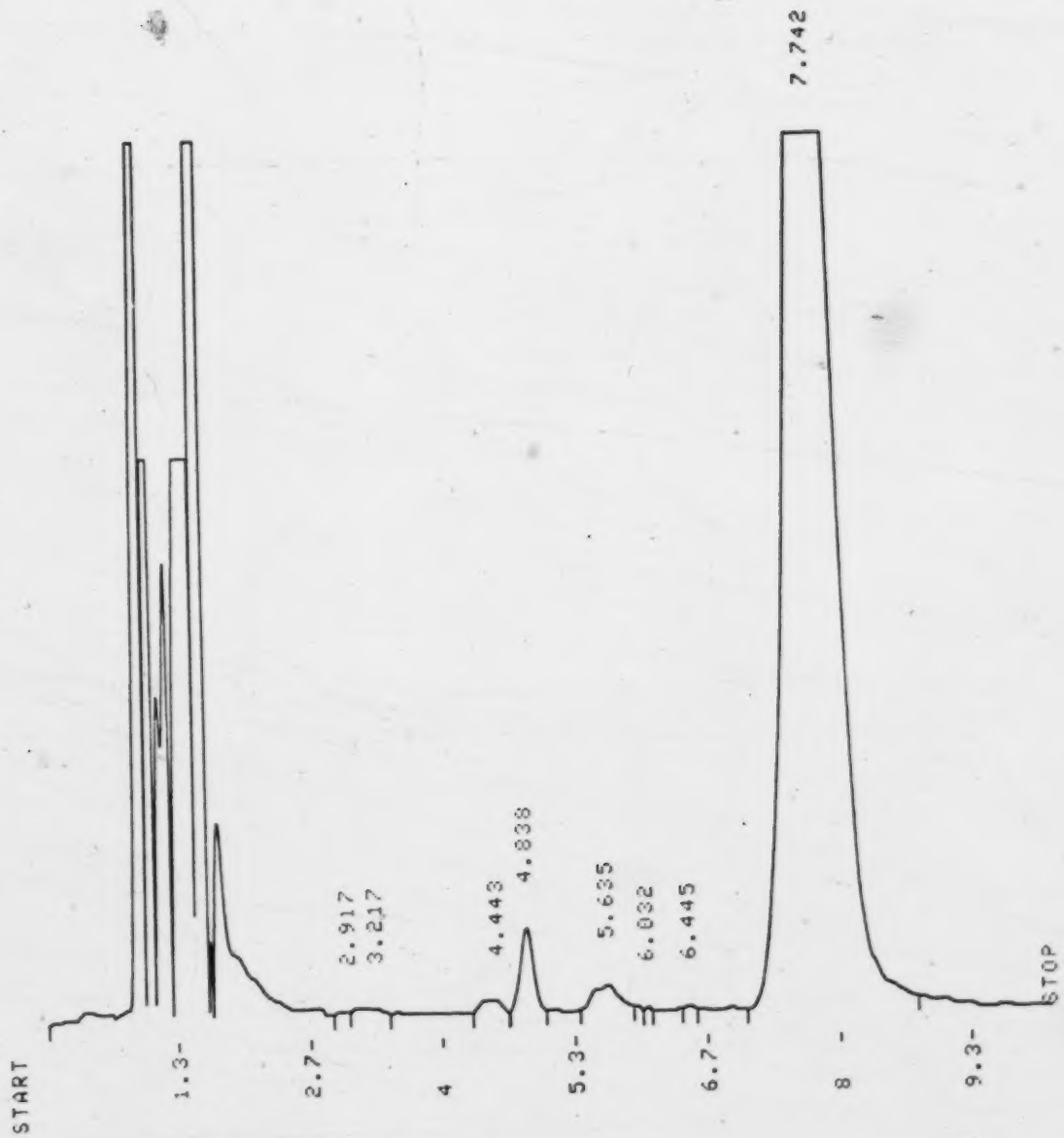
PKNO	TIME	HIGHT	MK	IDNO	CONC	NAME
3	2.357	52771		2	4298.3408	NOREPI
5	3.208	6877		3	816.8721	ADREN
7	4.445	18511		4	3195.2998	DOPAM
8	6.277	12523		1		EPININE
	TOTAL	90682			8310.5126	

Figure 28. Chromatogram of injection of standard catecholamines onto a deteriorating HPLC column.

important for the continuing operation and/or maintenance of the ECD at 1 nA F.S.D.

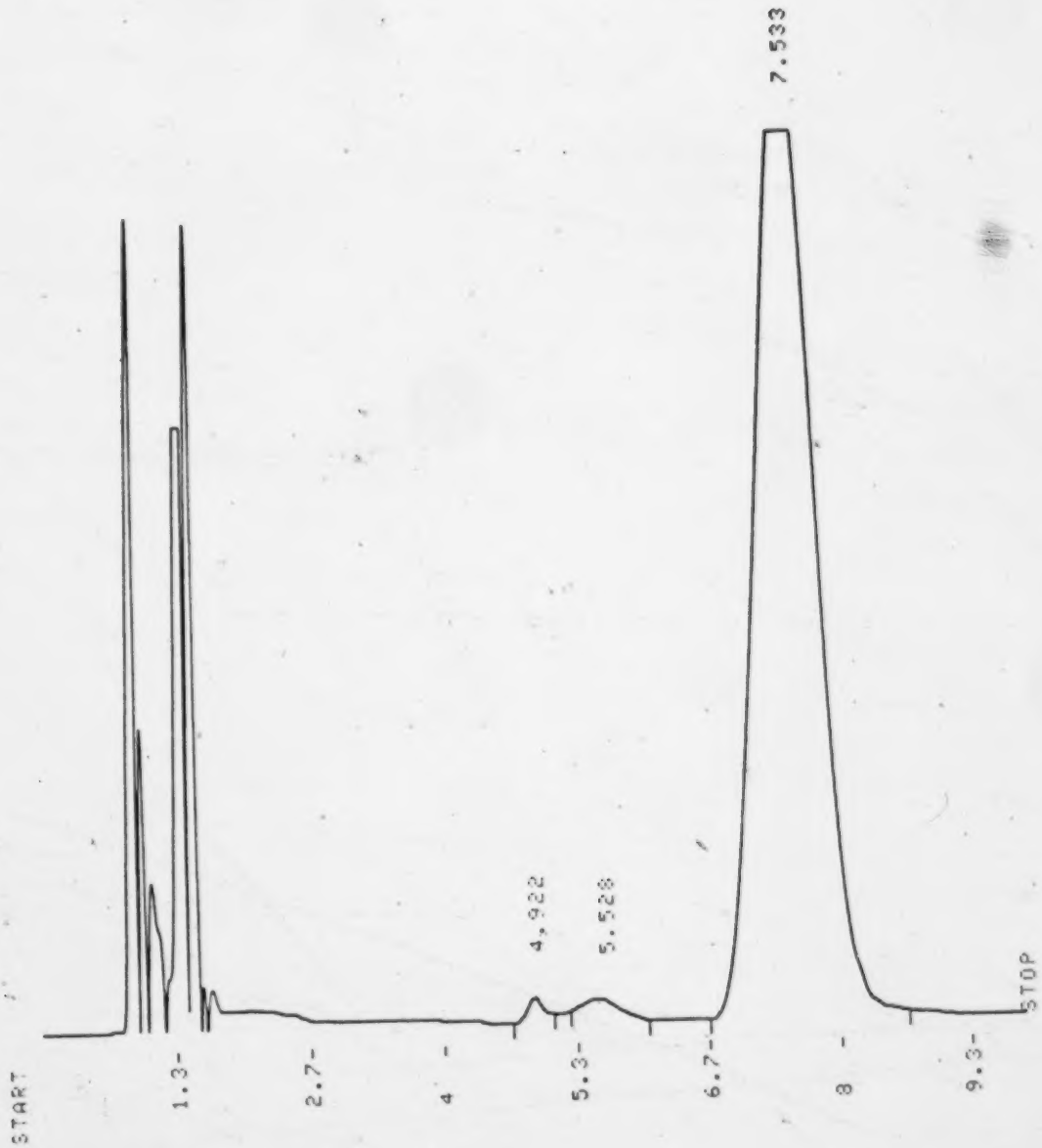
The gradual diminution in the sensitivity of the glassy carbon electrode is shown in Figures 29 to 33. This is a series of chromatograms generated by injecting 4000 pg DOE (in 50 µL 80 mM acetic acid) into the HPLC-ECD system over a period of time. The height of the DOE peak is 12,400 µV in Figure 29, 8,742 µV in Figure 30, 7,090 µV in Figure 31, 3,686 µV in Figure 32 and 2,913 µV in Figure 33. While the height of the DOE peak was diminished, the baseline in these chromatograms became progressively smoother thereby further indicating a loss of sensitivity. (The quiet baseline could have been mistaken for an improvement in the chromatographic conditions had it not been for the diminishing sensitivity of the WE).

The optimal method by which to achieve the restoration of the sensitivity of the glassy carbon electrode has been the topic of some controversy in the HPLC-ECD literature dealing with CA assays [e.g., Anton, 1984]. Until this time, it had been recommended that the surface of the glassy carbon electrode be polished with alumina. The assumption was made, but not specified, that this alumina suspension be composed of corundum. However, alumina suspensions were frequently ineffective in restoring the sensitivity of the electrode. Anton (1984) suggested an alternative method of polishing the cell namely, chromic acid, which, while effective, carried with it the risk of damage to the lucite. Additionally,



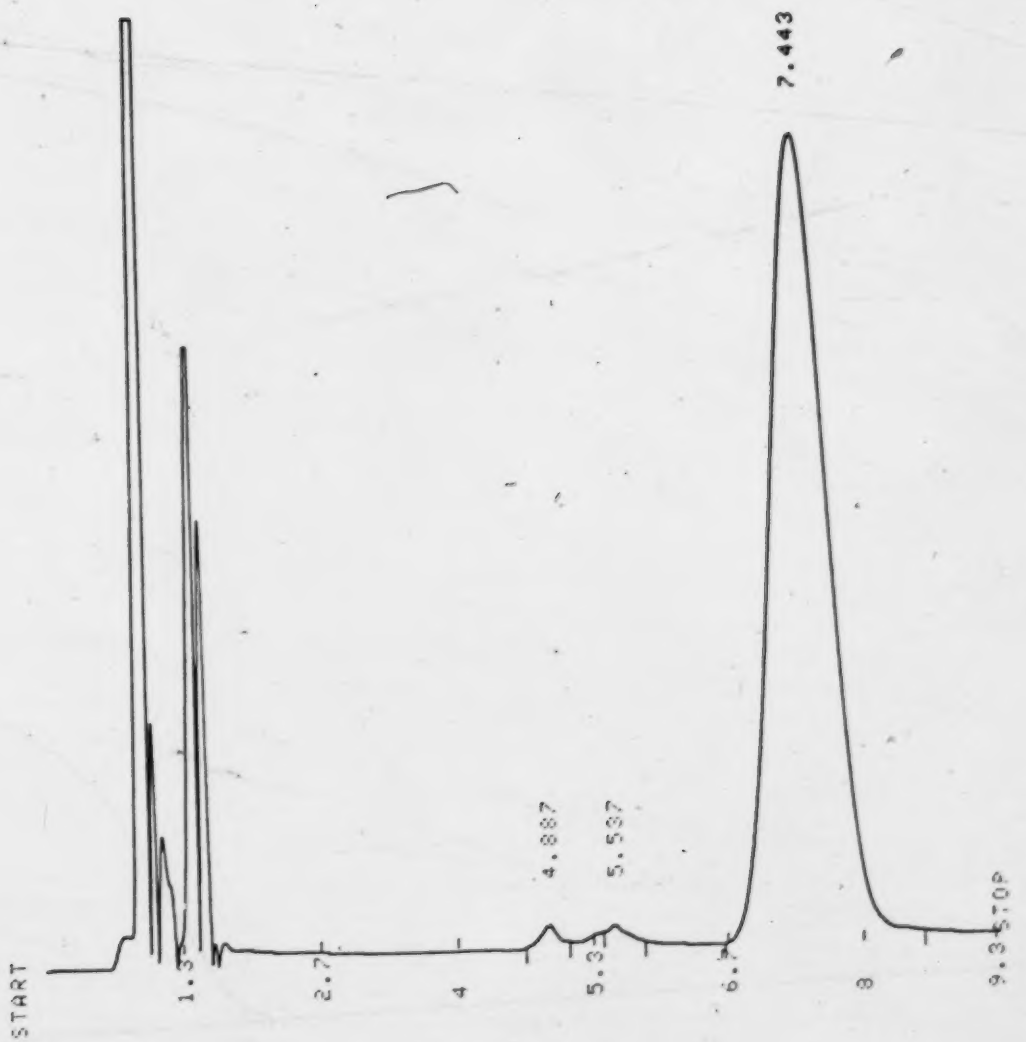
PKNO	TIME	HIGHT	MK	IDNO	CONC	NAME
4	7.742	12400		1		EPININE
	TOTAL	12400			0	

Figure 29. Chromatogram of a standard injection of deoxyepinephrine under near-optimal operating conditions.



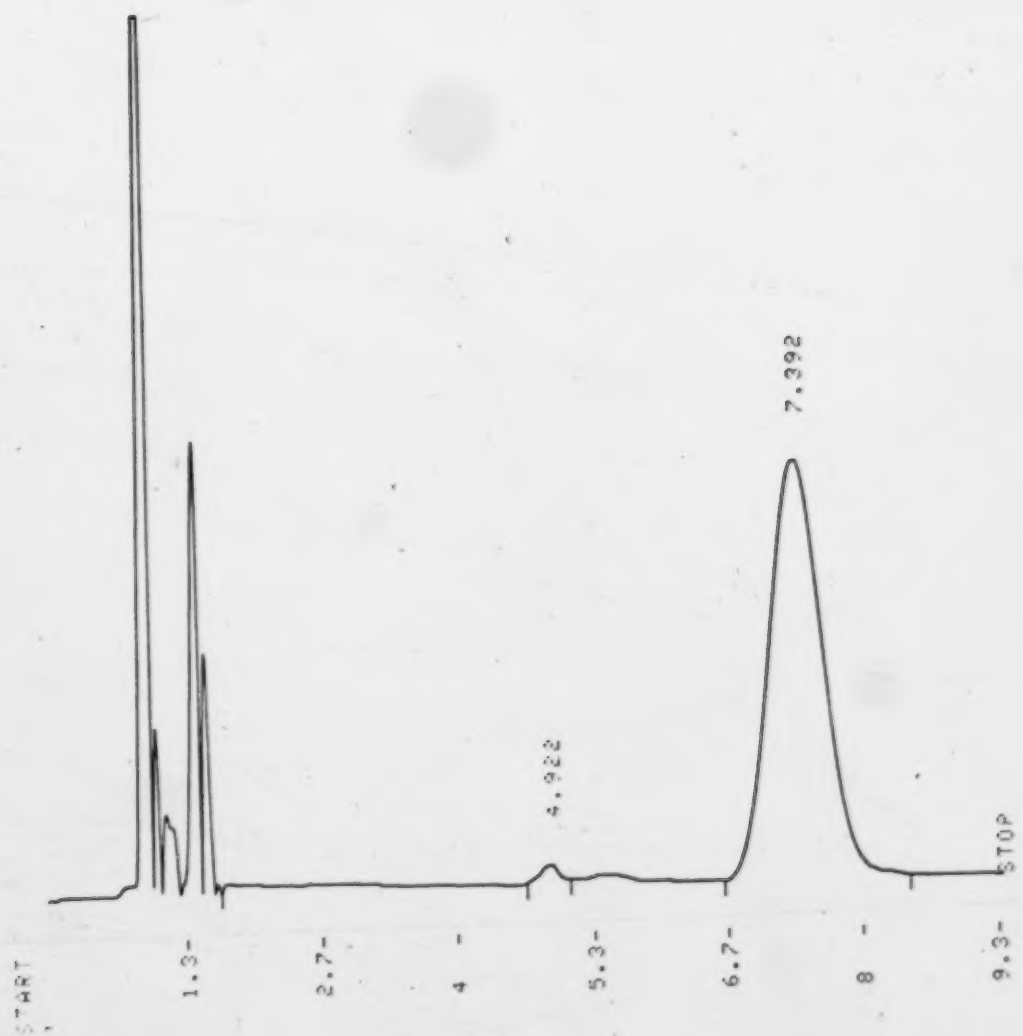
PKNO	TIME	HIGHT	MK	IDNO	CONC	NAME
2	4.922	190		4	62.8005	DOPAM
3	7.533	8742		1		EPININE
	TOTAL	8932			62.8005	

Figure 30. Chromatogram of a standard injection of deoxyepinephrine showing a diminishing ECD response.



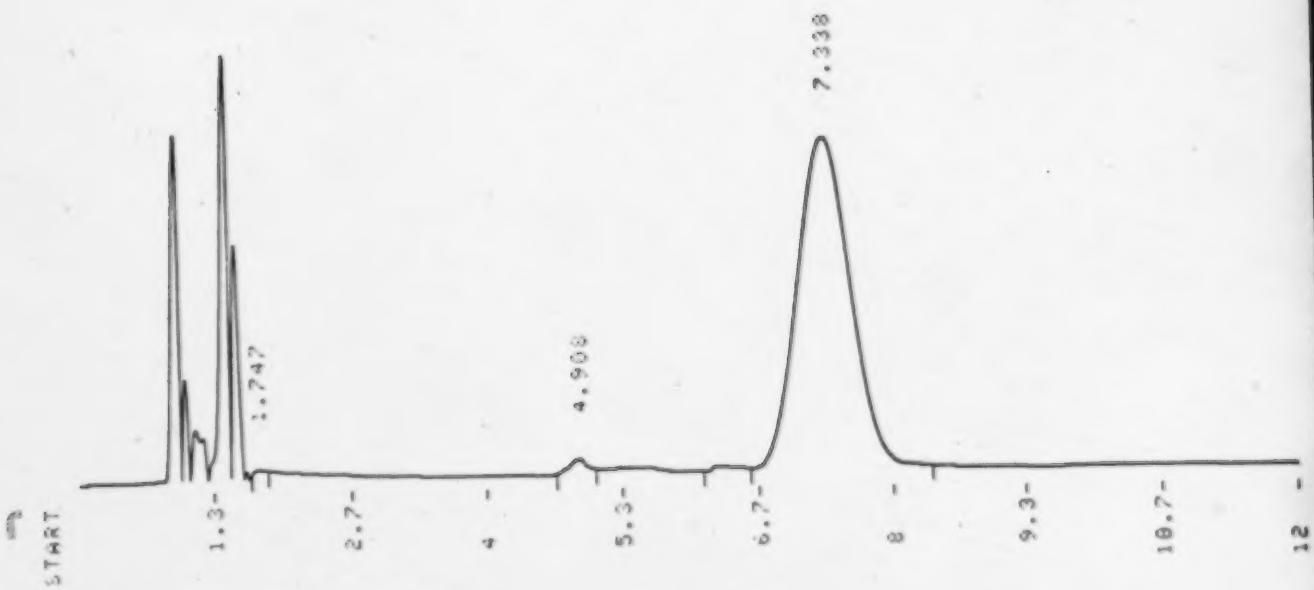
PEAK#	TIME	AREA	HT	IDENT	CONC	NAME
2	7.443	7090		1		EPININE
	TOTAL	7090			0	

Figure 31. Chromatogram of a standard injection of deoxyepinephrine showing further diminishing ECD response.



PKNO	TIME	-IG-T	TK	IDNO	CONC	NAME
1	4.922	151		4	118.5582	DOPAM
2	7.392	3686		1		EPIMINE
	TOTAL	3837			118.5582	

Figure 32. Chromatogram of a standard injection of deoxyepinephrine showing severely diminished ECD response.



PKNO	TIME	HIGHT	%	ID NO	CONC	NAME
2	7.338	2913				EPININE
	TOTAL	2913				

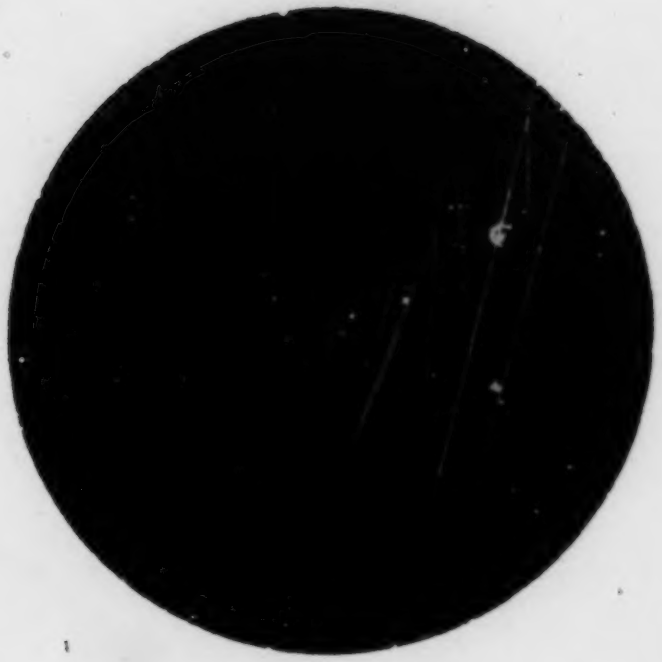
Figure 33. Chromatogram of a standard injection of deoxyepinephrine showing a blunted ECD response.

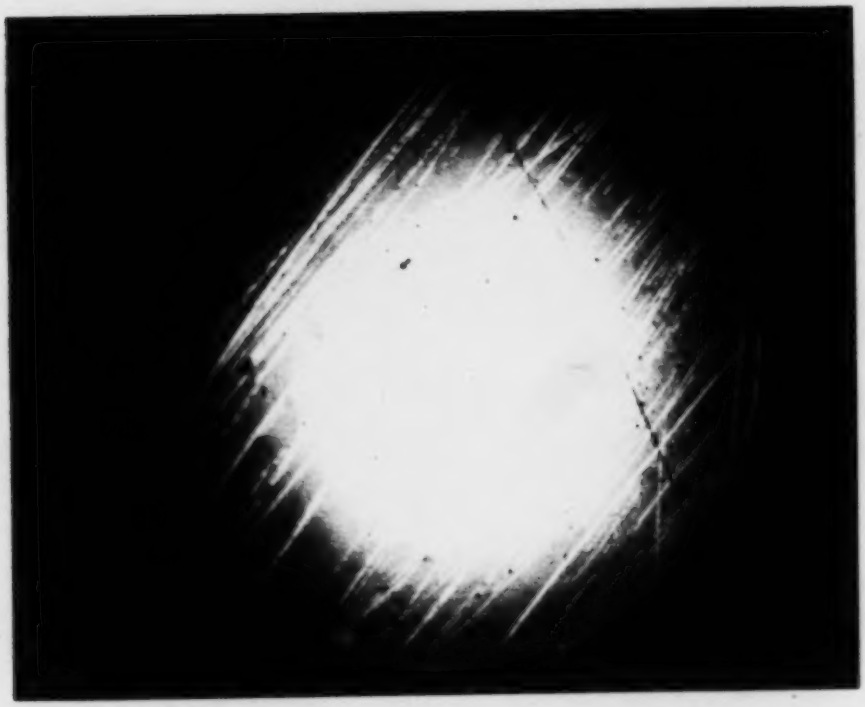
chromium ions are electroactive and if not removed completely, will participate in the electrochemical reactions occurring on the surface of the electrode.

In an effort to resolve this issue, photographs were taken of the surface of the glassy carbon electrode [Figure 34, 20x magnification]. It is apparent that the surface of the cell is marked with many fine lines running perpendicular to each other and across the cell surface. These lines are actually grooves ≤ 0.02 μm deep (as determined by a Talysurf 4 [Rank, Taylor, Hobson, Leicester, England] surface measurement instrument). The existence of these lines has not been described previously. It is also apparent that there are fine particles embedded in the grooves.

It was postulated that the fine particles seen in these photo-micrographs were composed of alumina particles left behind from the polishing of the cell by the conventional method namely, polishing the working electrode with a suspension of alumina particles supplied by the ECD's manufacturer. This alumina was assumed to be comprised solely of α-alumina (i.e., corundum). X-ray diffraction (XRD) analysis was performed (by Dr. R. Fiedorow of the Department of Chemical Engineering of this University) on this suspension using the method of Wanke & Fiedorow, 1988, to determine whether this was, in fact, the case. The XRD studies were performed on a monochromatic (graphite), X-ray diffractometer (Philips, The Netherlands) equipped with a copper tube. The diffractometer was operated in the step-scan mode @ 0.2 °2θ

Figure 34. Photomicrographs of the surface of two glassy carbon working electrodes from the ECD.





per step and counted for 100 sec/step. The total amount of time required for the analysis of the alumina sample was 11.7 hours. The XRD patterns obtained (i.e., angle-intensity data) were processed by an on-line mini-computor and plotted as intensity (c/s) versus diffraction angle (2θ).

The results of the XRD analysis are shown in Figure 35. These data indicate that the suspension used to polish the glassy carbon working electrode was comprised of a mixture of α- and γ-alumina.

In anticipation of these data, the polishing methods described in section A.4.(F) of Methods were adopted several years ago in an effort to circumvent the confounding effects (described in Discussion Section, below) of the γ-alumina.

C. Subarachnoid Hemorrhage Patient Study

Five patients with documented SAH due to rupture of a cerebral artery aneurysm were ultimately selected for participation in this study of the contribution of the plasma CA levels to the development of vasospasm. The basic clinical data on these patients are reported in Table 8. The study group comprised three middle-aged women and two young men. The CA profiles of these patients throughout the course of their participation in this study are portrayed graphically (along with concurrent physiological parameters) in Figures 36 to 40. All patients were operated on (i.e., their aneurysms were clipped) within two days of entering the CA study.

Figure 35. X-ray diffraction pattern for alumina supplied to polish the glassy carbon working electrode of the ECD.

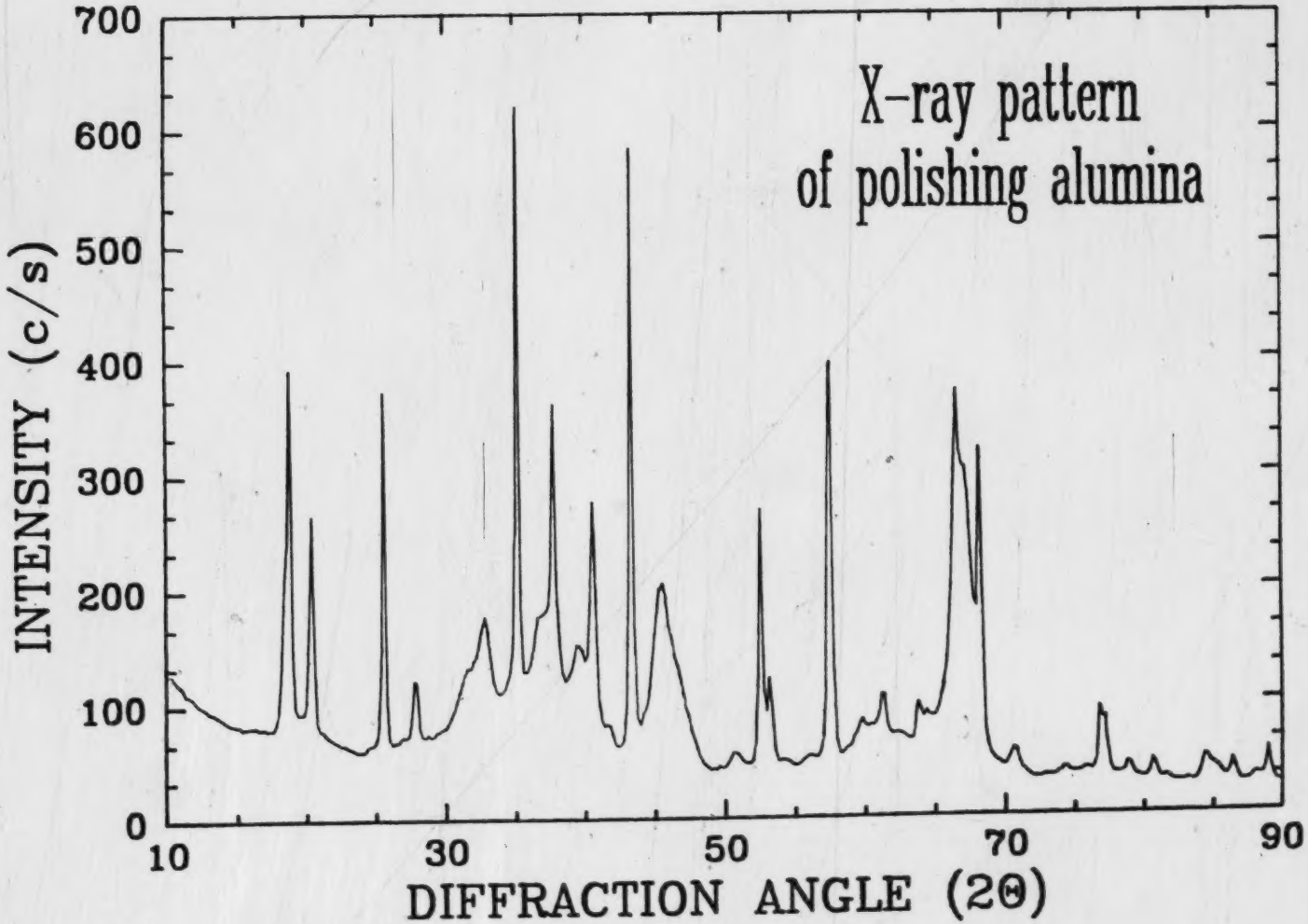

X-ray pattern
of polishing alumina
INTENSITY (c/s)
700
600
500
400
300
200
100
0
10
30
50
70
90
DIFFRACTION ANGLE (2Θ)

Table 8. Clinical data for good-grade SAH patients.

Sex	Age	Location of Aneurysm	Time of First Blood Sample Post SAH (hours)	Days in CA Study	Post-Op Spasm
M	25	Post. Comm. Artery	12	10	No
M	24	Rt. Mid. Cer. Art.	24-48	9	No
F	52	Rt. Post. Comm. Art.	18	6	No
F	59	Rt. Post. Comm. Art.	96	8	No
F	57	Ant. Comm. Art.	56	14	Yes

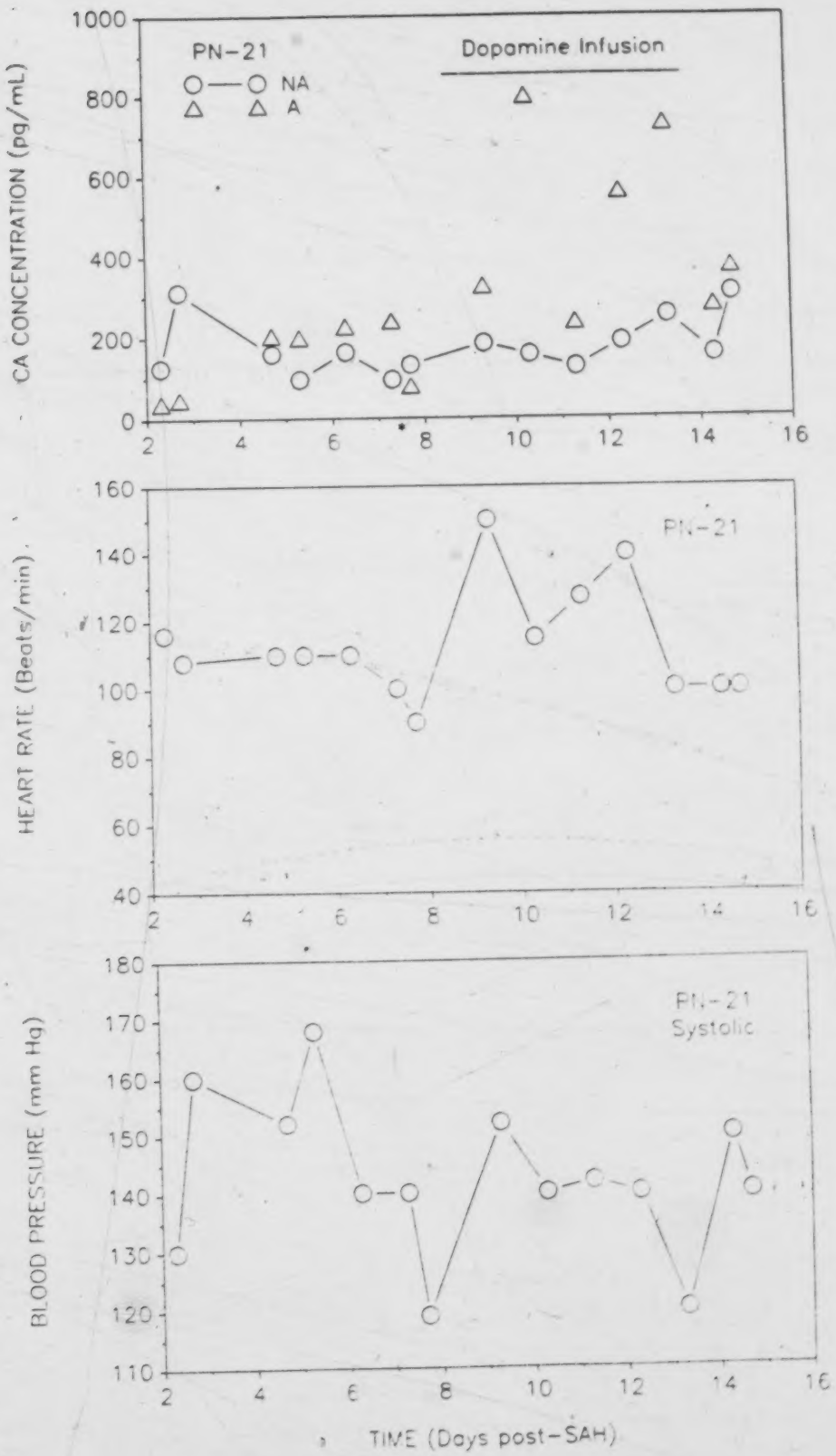
Where:
Post. Comm. Art. = Posterior Communicating Artery
Rt. Mid. Cer. Art. = Right Middle Cerebral Artery
Rt. Post. Comm. Art. = Right Posterior Communicating Artery
Ant. Comm. Art. = Anterior Communicating Artery.

The patients, as listed from top to bottom in this table are: PN-06, PN-15, PN-08, PN-17, PN-21.

Only one of these patients (PN-21, 57 year old female) [Figure 36] developed cerebral artery vasospasm. She was admitted to hospital approximately two and a half days post-SAH with a Glasgow Coma Score (GCS) of 15. However, post-operatively she was unresponsive (GCS had declined to 7 and was not a side-effect from morphine or valium) and continued to deteriorate neurologically. Vasospasm was documented angiographically on the seventh day post-SAH and shortly thereafter, she started to receive an infusion of DA to counteract her reduced blood pressure (i.e., within 12 hours of the angiogram). The plasma NA concentrations attained throughout the time of study of this patient were within the physiological range of normal for resting plasma samples in spite of the DA infusion. The high levels of plasma A noted during the second half of the period of study were most probably an artefact due to the dopamine infusion.

Subject PN-06 (25 year old male) arrived in hospital within 24 hours of suffering a SAH. His GCS was stable at 15 throughout the course of his stay in hospital and his clinical progress was uneventful. Throughout the course of the time of study, his plasma CA concentrations fluctuated from 0.5 to 2 to 3 times his plasma CA levels at admission to hospital [Figure 37] but remained within the physiological range of normal for resting plasma samples. On days 7, 8 and 9 post-SAH, his plasma CA levels increased sharply but the patient was complaining of sleep deprivation and headache at this time

Figure 36. Catecholamine concentrations and physiological data for SAH patient PN-21.


PN-21
Dopamine Infusion
NA
A
CA CONCENTRATION (pg/mL)
1000
800
600
400
200
0
2
4
6
8
10
12
14
16
HEART RATE (Beats/min)
160
140
120
100
80
60
40
PN-21
BLOOD PRESSURE (mm Hg)
180
170
160
150
140
130
120
110
PN-21
Systolic
TIME (Days post-SAH)

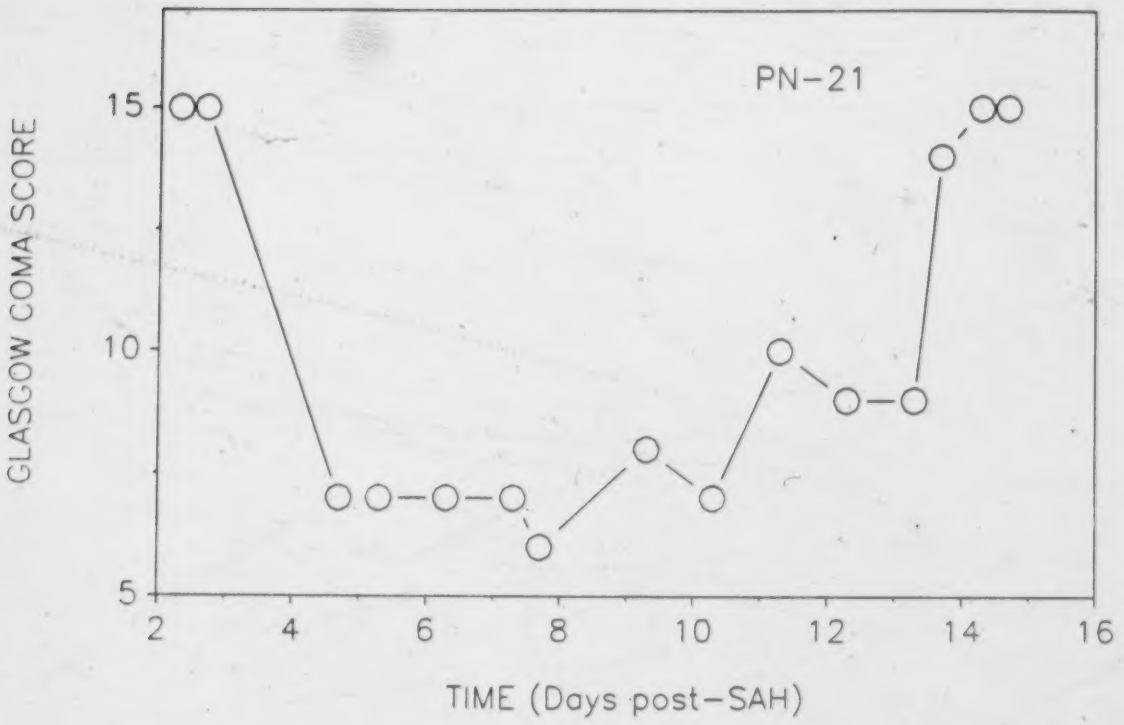
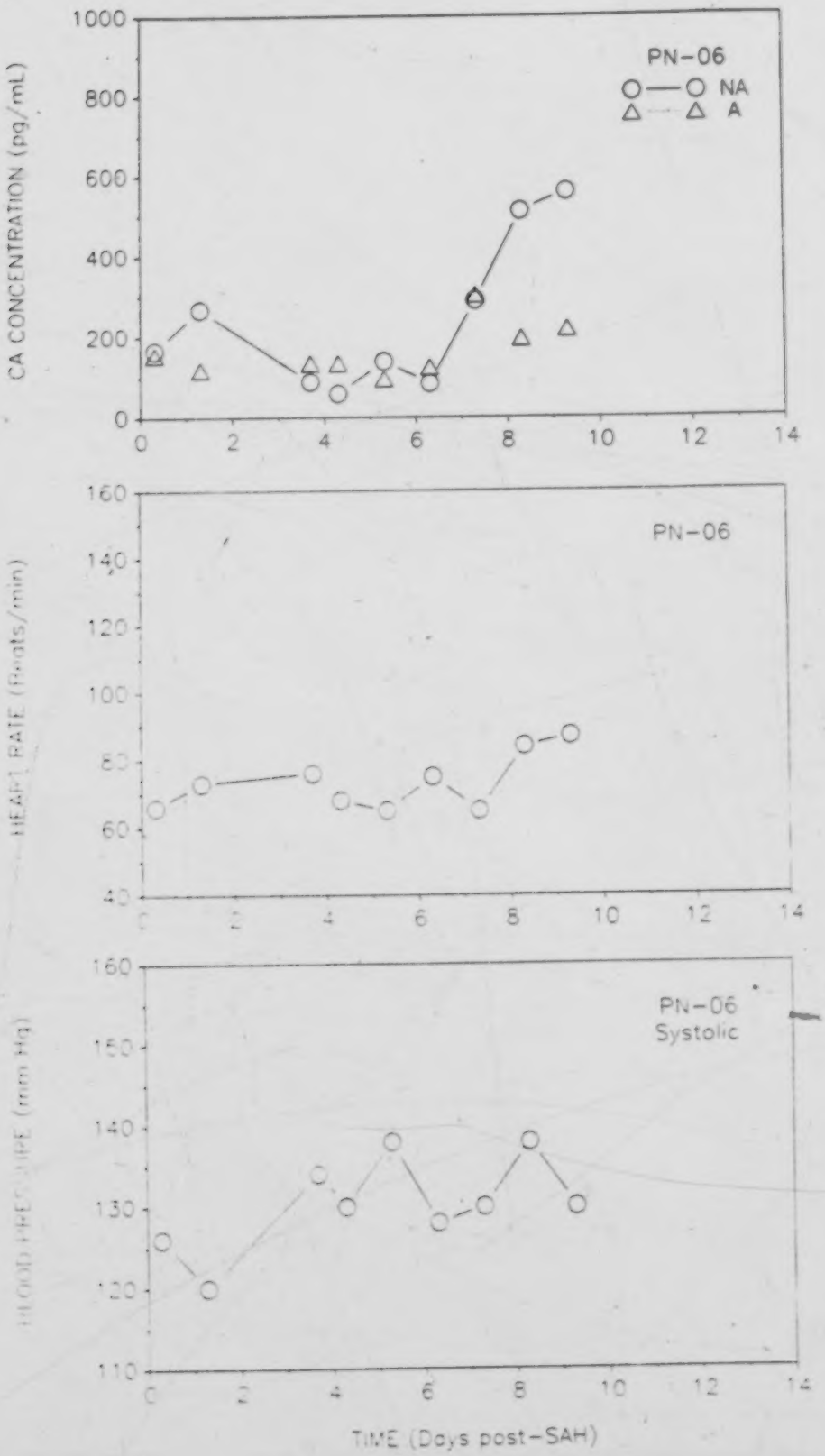


Figure 36. Cont'd...

Figure 37. Catecholamine concentrations and physiological data for SAH patient PN-06.


PN-06
NA
A
CA CONCENTRATION (pg/mL)
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
PN-06
HEART RATE (beats/min)
160
140
120
100
80
60
40
PN-06
Systolic
160
150
140
130
120
110
TIME (Days post-SAH)

and this factor must be taken into consideration during interpretations of his plasma CA levels.

Subject PN-15 arrived in hospital within 24-48 hours of suffering a SAH, in a state of confusion sufficient to lower his GCS to 13 for the first two days of his stay in hospital. However, this returned to a GCS score of 15 within two days of surgery. His clinical course was uneventful and he was discharged without further physiological complications. Throughout the course of his participation in this study, his plasma CA levels fluctuated from 0.5 to 2 to 5 times his plasma CA levels at admission [Figure 38] but remained within the physiological range of normal for resting plasma samples.

Subject PN-08 (52 year old female) was admitted to hospital within 18 hours of having suffered a SAH and maintained a GCS of 15 throughout the course of her stay in hospital. Her clinical course was uneventful; her plasma NA levels fluctuated from 0.5 to 2 times her plasma levels at admission, and her A levels stayed at or below what they were on admission [Figure 39]. All of these apparent fluctuations in plasma CA levels, however, were within the physiological range of normal for resting plasma samples.

Subject PN-17 (59 year old female) was admitted to hospital within 96 hours of suffering a SAH, with a GCS of 15 which she maintained throughout the course of her stay in hospital. Her clinical progress was uneventful. Her plasma CA levels were from 0.5 to 2 times admission values but

Figure 38. Catecholamine concentrations and physiological data for SAH patient PN-15.

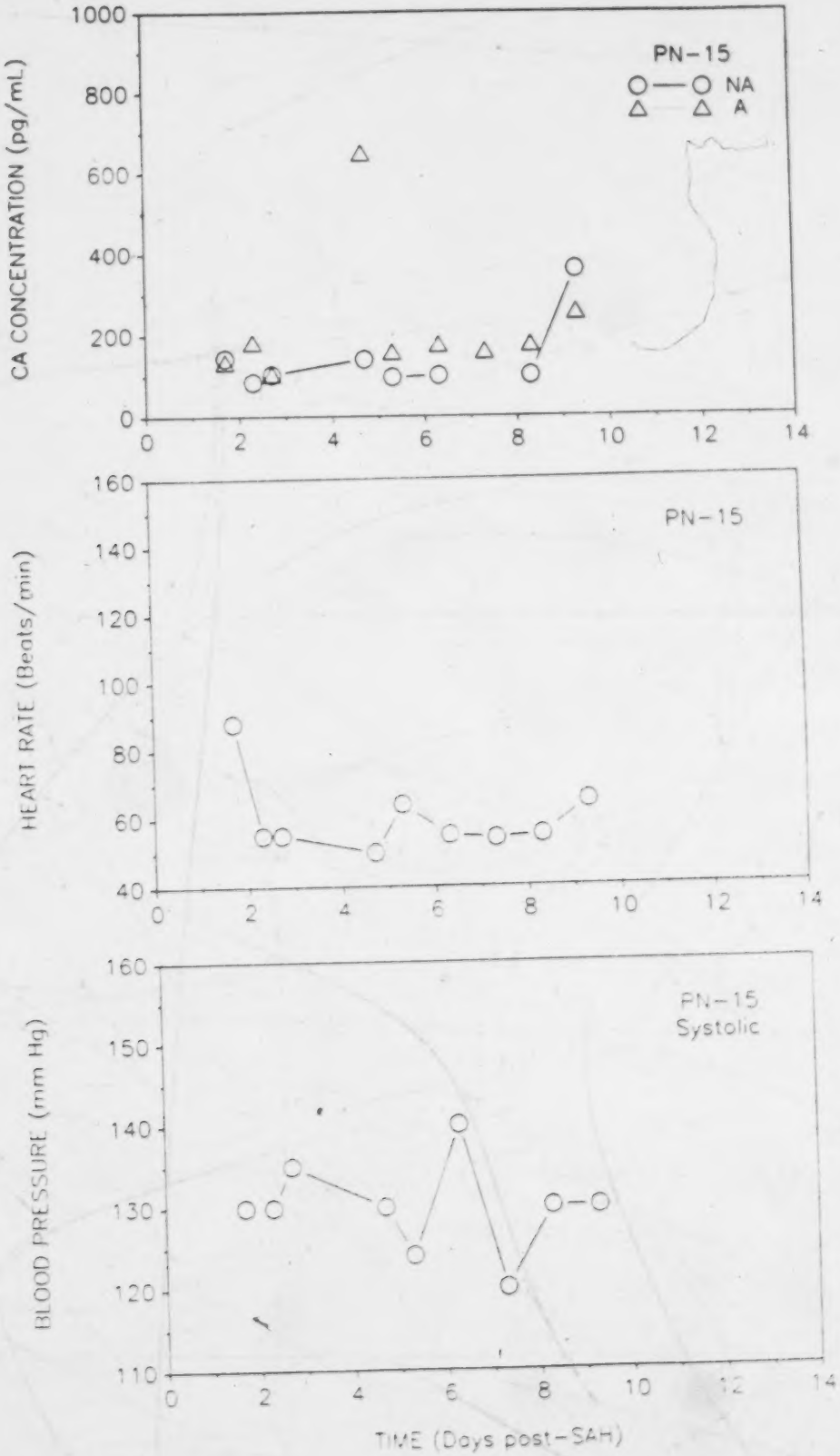
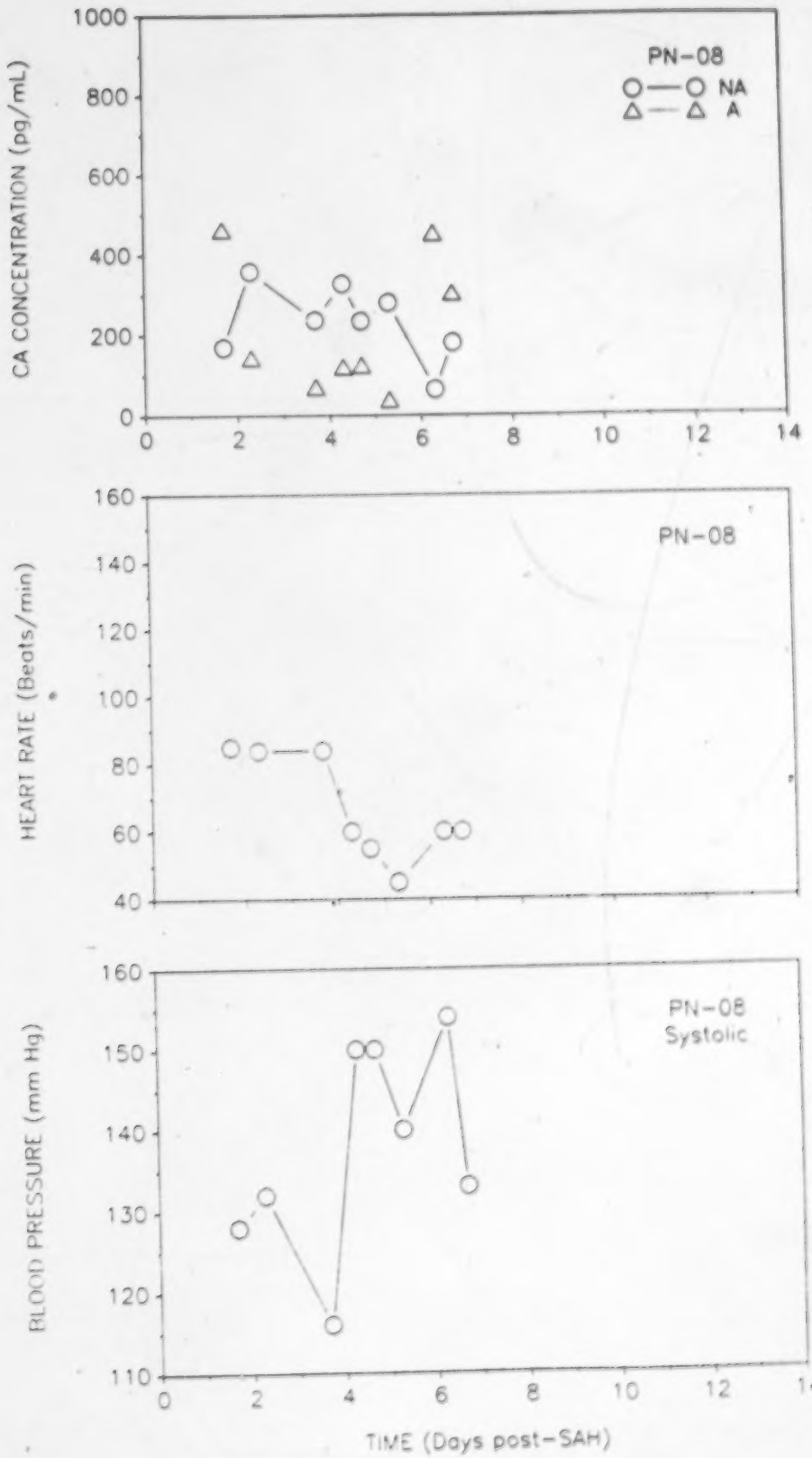

PN-15
NA
A
CA CONCENTRATION (pg/mL)
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
PN-15
HEART RATE (Beats/min)
160
140
120
100
80
60
40
PN-15
Systolic
BLOOD PRESSURE (mm Hg)
160
150
140
130
120
110
TIME (Days post-SAH)

Figure 39. Catecholamine concentrations and physiological data for SAH patient PN-08.


PN-08
NA
A
CA CONCENTRATION (pg/mL)
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
PN-08
HEART RATE (Beats/min)
160
140
120
100
80
60
40
PN-08
Systolic
BLOOD PRESSURE (mm Hg)
160
150
140
130
120
110
0
2
4
6
8
10
12
14
TIME (Days post-SAH)

remained within the physiological range of normal for resting plasma samples [Figure 40].

The data from the comparison study of the two sisters (aged 48, PN-08S, and 52, PN-08) both of whom developed SAHs within six months of each other are shown in Figure 41. The sister, PN-08S, had an aneurysm of the right posterior inferior cerebellar artery, and her blood samples were taken starting on the eighth day post-SAH and continuing through four post-operative days (i.e., until the fifteenth day post-SAH). Her clinical progress was uneventful except for a brief (i.e., four hours long) hypotensive episode on the same afternoon as the first blood sample that was taken for CA analysis.

Although these two patients (i.e., PN-08S and PN-08) had very different clinical management (i.e., delayed versus immediate surgery, respectively, to clip the aneurysm), they both had uneventful clinical courses and neither of them developed vasospasm. Furthermore, neither of them had plasma CA levels elevated beyond the physiological range of normal for resting plasma samples.

Figure 40. Catecholamine concentrations and physiological data for SAH patient PN-17.

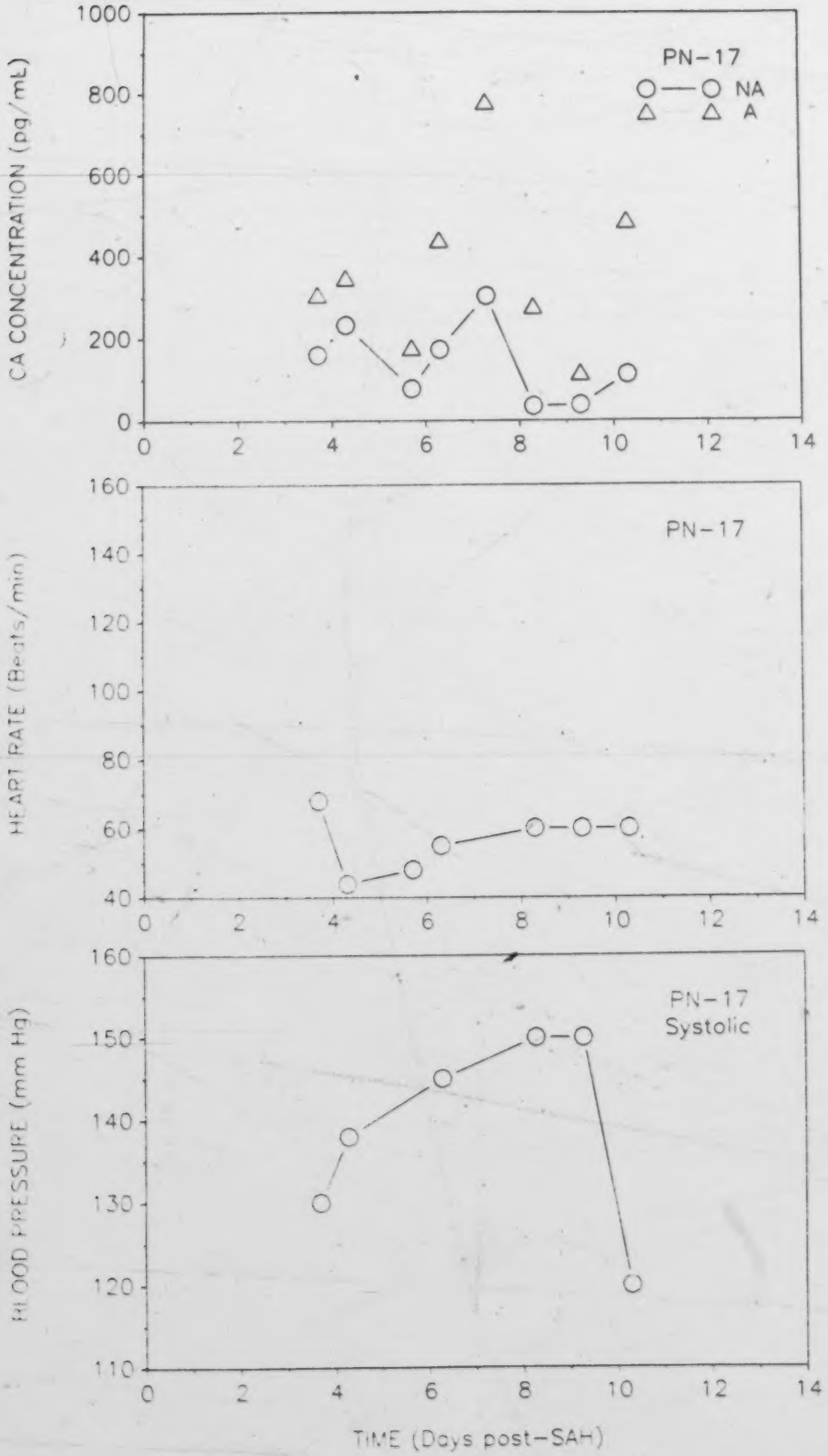
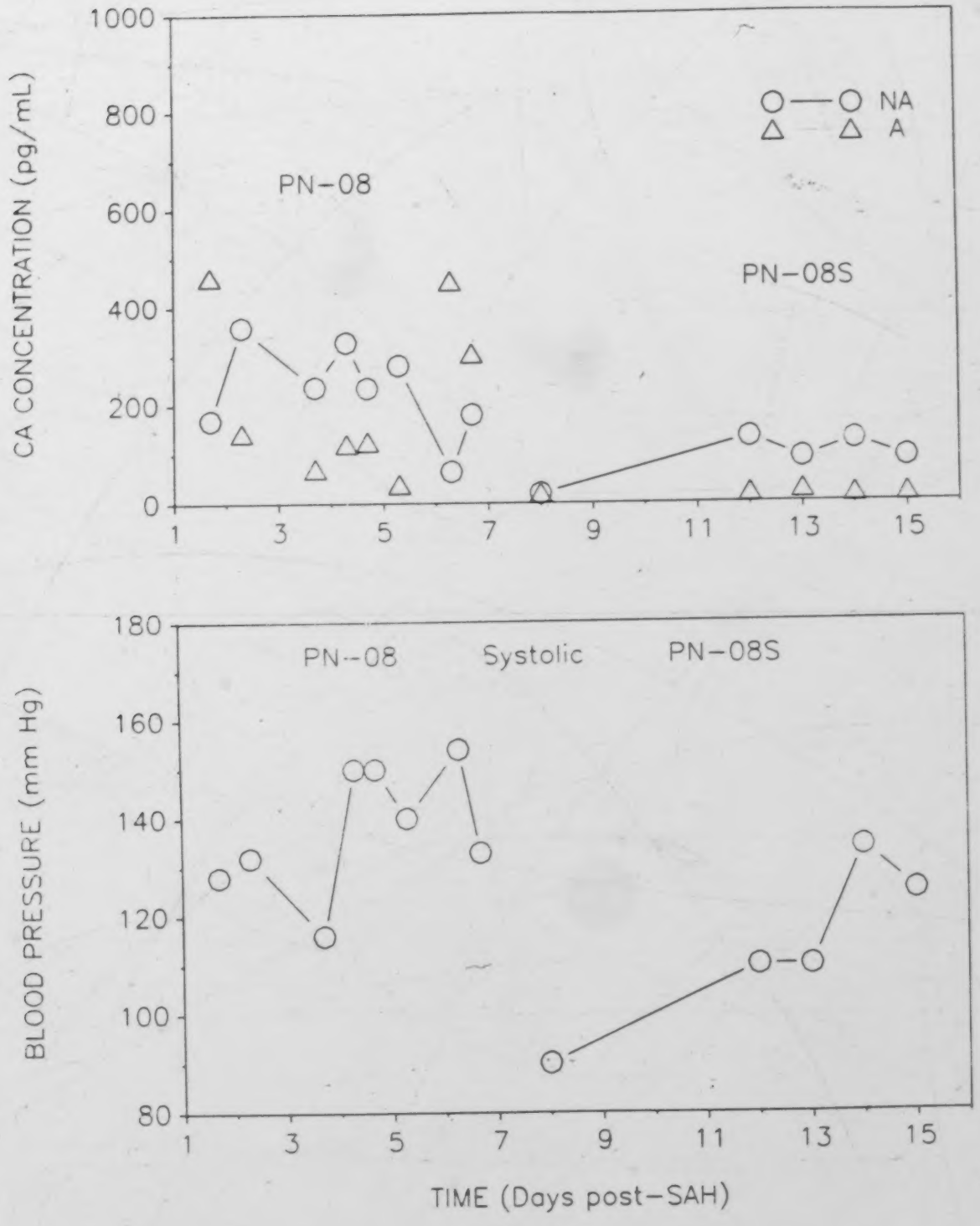

CA CONCENTRATION (pg/mL)
1000
800
600
400
200
0
PN-17
NA
A
0
2
4
6
8
10
12
14
HEART RATE (Beats/min)
160
140
120
100
80
60
40
PN-17
BLOOD PRESSURE (mm Hg)
160
150
140
130
120
110
PN-17
Systolic
TIME (Days post-SAH)

Figure 41. Catecholamine concentrations and physiological data for SAH patients PN-08 and PN-08S.


CA CONCENTRATION (pg/mL)
1000
800
600
400
200
0
NA
A
PN-08
PN-08S
1
3
5
7
9
11
13
15
BLOOD PRESSURE (mm Hg)
180
160
140
120
100
80
PN--08
Systolic
PN-08S
TIME (Days post-SAH)

DISCUSSION

The human plasma CA assay continues to be one of the most problematic of analyses to master. The analytical significance of the fact that the CA are themselves labile and unstable molecules, coupled with the problem that the CA are of low molecular weight and present only in very minute quantities in plasma have insured the endurance of this analysis as one of the "Holy Grails" of the clinical chemistry laboratory.

No single, optimal method of assaying human plasma CA has emerged to date. There are almost as many CA assays in the literature as there are laboratories performing the analysis [Hjemdahl, 1984b]. The promise of HPLC-ECD technology has made the HPLC-ECD CA assay the method of choice recently. However, the accurate quantification of CA by HPLC-ECD has not proven to be facile largely because this assay has taxed, to the limits, the technology comprising the HPLC-ECD apparatus.

The capabilities of HPLC-ECD to separate and quantify CA of human plasma have also been confined by the special properties of the CA themselves (i.e., physicochemical properties) and limited by the complexity of the plasma containing the CA to be quantified. [Plasma is not the primary vehicle for CA but rather only contains the spillover of CA released by sympathetic neurons (See Introduction)]. These problems have precluded the development and

implementation of simple, sensitive and reliable CA assays for routine use in clinical laboratories.

The discussion to follow includes a consideration of some of the technical difficulties associated with the HPLC-ECD CA assay and an examination of how these problems have been solved for the assay reported. The discussion will be presented in two parts: Part A will deal with the implications of the HPLC-ECD CA assay and will include (1) a consideration of the choice of equipment used and operating conditions chosen, (2) an examination of the problem of extraction of CA from plasma, (3) the validation of the method reported, and (4) a review of the issues involved in the maintenance of this CA assay; and Part B will address the issue of the clinical application of the CA assay technique from the point of view of (1) data obtained in the present study and (2) a comparison with other CA studies of SAH patients reported in the literature. The discussion will be concluded (C) with a summary of the value of the present investigation.

A. Implications of the HPLC-ECD CA Assay

The present CA assay has been optimized for functioning of the HPLC-ECD apparatus at a sensitivity level of 1 nA F.S.D. on the ECD [Damjanovic & Russell, 1986]. This provides a very sensitive assay (see below) and is unique in that this sensitivity level was maintained over relatively long periods of time for the quantification of normal plasma

concentrations of CA. During the development of this assay, it became apparent that the utility and success of the entire assay procedure depended on having continual access to this level of sensitivity. However, not only was a sensitive response of the ECD to the CA necessary, but also a stable and "quiet" baseline on the chromatogram was essential. This, in turn, depended, quite literally, on the state of the apparatus (as manifested by the baseline signal produced and the signal:noise ratio).

Stated simply, the usefulness of the HPLC-ECD apparatus for plasma CA assays depends on two main factors, the sensitivity of the ECD and the stability of the baseline output of the ECD (signal:noise ratio). The baseline output may determine the level of sensitivity useable for the CA assays.

For the above reasons, in 1984, the development of the present assay became focussed on the reduction of the amount of baseline noise present at 1 nA. This concept had not previously been used as a point of focus for HPLC-ECD CA assay development and it provided novel insights into the equipment and the functioning of the entire assay. All procedures that were performed in the laboratory to support the assay were re-examined in light of their effect on the apparatus at this high sensitivity level. For example, the electroactivity of all chemical contaminants became important if there was any chance that these contaminants might persist through the apparatus and come into contact with the column and/or

detector cell surface. Furthermore, the chemical structures of compounds which came into contact with the extraction procedure were evaluated for possible interference with either the CA themselves or possible interaction with the column and/or the cell. The use of the Shimadzu integrator facilitated this investigation because it gave an objective characterization of the output signal of the ECD. Because the ECD was set at 1 nA, interferences and sources of baseline noise were readily and immediately apparent. In this way, the roles of electrical grounding, leaking compression fittings, in-line filters, chemical contaminants and so on were elucidated. While many of these observations are not unique, per se, the identification of their contribution to the optimal functioning of the HPLC-ECD apparatus is novel.

The reason for the requirement of optimal functioning at 1 nA F.S.D. is as follows:

Resting plasma CA concentrations (background or control concentrations in resting humans) are approximately 150 to 300 pg/mL NA, 20 to 75 pg/mL A and perhaps 0 to 100 pg/mL DA. An injection into the HPLC-ECD of a standard solution of CA containing two or three times these resting values yields peaks of the following sizes at 1 nA F.S.D.: 500 pg NA gives rise to a peak of only approximately 65% F.S.D.; 100 pg A gives a peak of only 9% F.S.D.; and 200 pg DA gives a peak of only 9% F.S.D. If the chart paper is 12.5 cm wide and the baseline is 0.5 cm from the edge of the paper, a 10% F.S.D. is a peak, at best, only 1.2 cm high. Therefore, a 25% reduction

in the size of this peak could render it only marginally distinguishable from baseline noise. And, excessive baseline noise would make accurate measurement of this peak impossible.

Even if the plasma contained the relatively high concentrations of 6000 pg/mL NA and 1000 pg/mL A, extraction of 2 mL of plasma into 250 µL of acetic acid and injection of 50 µL of that would place only 400 pg of A on the column. At 2 nA F.S.D., this would result in a peak height of only 2 cm (the 2400 pg of NA would be off-scale; this NA concentration correponds to a peak height of 65% F.S.D. at 5 nA). Hence, for A, a setting of 1 nA F.S.D. would be prefered in this case as well. The Shimadzu integrator can deal with peaks that are off-scale but it cannot enlarge small peaks.

The above examples of peak heights resulting from plasma concentrations of CA in normal, resting humans or normal, exercising humans, respectively, indicate the need for high sensitivity in the HPLC-ECD system. Even at 1 nA F.S.D., all other aspects of the system should be near-optimal for accurate quantification of NA and especially A. As demonstrated in the polarograms, the difference in electroactivity between NA and A makes A more difficult to quantify by the ECD method (because A is less electroactive than NA). A further complication of this matter resides in the fact that the physiologically relevant concentration range of A is considerably lower than that of NA (i.e., there is relatively little A to detect). Obviously, a plasma

extraction technique which is inefficient will jeopardize the ability to detect A at all.

It is important to appreciate that the range setting on the ECD is merely an attenuator for the output of the ECD cell. That is, the baseline stability of the signal emanating from the detector cell at a range setting of 5 nA is considerably greater than the stability of the cell at 1 nA. Therefore, it is the range setting which can be reasonably maintained with a workable signal:noise ratio that will determine the functional sensitivity of the ECD and hence, of the HPLC-ECD apparatus.

1. Consideration of the Choice of Operating Conditions

(a) HPLC-ECD Apparatus

Amperometric ECDs suitable for biomedical analyses have been commercially available since approximately 1978. The original thin-layer, flow-through, carbon paste ECD cell was the subject of a Ph.D. thesis (Kissinger, 1973). Experience with the detectors has led to further improvements in the hardware that makes up the detector assembly and electronic controls. For example, the stainless steel top for the thin-layer cell became commercially available in 1984 and the active filter kit for the ECD electronics became available in 1985.

The evolution of the HPLC technology continued in parallel with improvements to the ECDs [Molnar & Horvath 1976, 1978; Krstulovic, 1982; Mefford, 1987]. CA assay

protocols, in general, have incoporated new developments in the HPLC equipment as these have become commercially available [e.g., Smedes et al., 1985]. These new developments have included both obvious and subtle changes to the HPLC equipment. An example of the latter is the design and manufacturing of Teflon (instead of rubber) gaskets for the pump head seals. Teflon gaskets are inert in the acidic (or basic) MPs that are normally used in reverse-phase HPLC and do not disintegrate on contact with the MP. Teflon was also formed into a number of the components of the injector of the HPLC-ECD system for the same reason. By 1983, Teflon gaskets were readily, if not widely, available and injectors constructed by conscientious manufacturers incorporated Teflon components at strategic locations along the flowpath of the MP.

An example of a more obvious improvement in HPLC hardware was the creation and use of dedicated microprocessors connected to the HPLC-ECD system and capable of both programmed control of the system and recording and analyzing the data that ensued. This development introduced the possibility for the automation of changes to MP composition (e.g., for the purpose of gradient elution) and pump speed both during, and between, chromatographic runs. Furthermore, these mini-computors introduced the possibility of objective data collection, thus eliminating the biases of chromatographic peak height/area measurements conducted with a straight-edge ruler. Another benefit which accrued from the

use of these microprocessors was the opportunity to preserve data and re-analyze it as necessary. For this reason, a dedicated integrator (Shimadzu C-R3A) was added to the CA assay method (described herein) in 1985.

One important aspect of the HPLC apparatus was the fact that it was operating at high pressures (e.g., 3000 psi). For the sensitive CA assay, a leak-free system is virtually essential. While the recommendations for the maintenance of leak-free connections along the flowpath of the MP existed, the complete implications (i.e., the consequences) of not doing so were not emphasized. When an HPLC apparatus is purchased, the buyer is supplied only with a metal file (or else a very slightly more sophisticated "tube cutter") to create new cuts for pieces of tubing.

Leakage problems and baseline noise were routinely encountered during the early development of the current assay. Therefore, in an effort to associate and diminish these phenomena, the actual condition of the ends of the stainless steel tubing (sampled from a variety of locations along the flowpath of the MP) was investigated by micro-photography. It is apparent from the photographs that only professionally cut tubing with perfect ends is ideally suitable (demonstrated in Figure 7). This information is unique and this issue has not been specifically addressed in the CA assay literature. Only one scientific supply company (Rainin, Woburn, MA.) offers pre-cut and pre-finished tubing but the availability of these items has not been advertised by suppliers of HPLC apparatus.

(Copies of these photographs were sent to Waters Scientific Co. in Toronto; a company representative admitted that the Company had not previously had photographic evidence of this well-known problem of HPLC apparatus.)

The use of the Faraday cage, additional pulse dampening in the flowpath of the apparatus, addition of in-line filters and the use of helium gas to purge the MP, inclusion of the stainless steel ECD cell top and the active ECD filter kit described herein all contributed to the maintenance of 1 nA F.S.D. However, while these are not unique practices, they are also not universal especially when used in combination.

Another important problem that was solved by the assay reported herein was the question of the nature of the surface of the glassy carbon electrode. It had been believed that the surface of the WE was "mirror-like" [BAS LC-4B Operator's Manual, 1982; Hjemdahl, 1987]. The establishment of the fact that the cell surface is not "mirror-like" is novel and has a variety of implications. However, the grooves in the glassy carbon WE are still shallower than the size of the grooves previously reported for the carbon paste WE (i.e., 0.02 vs. 2.0 μm, respectively) [Fenn et al., 1978]. The significance of these facts includes the possibility that the grooves on the surface of the WE provide a place for particles (such as catalytic alumina particles) to be deposited. Furthermore, the existence of the grooves indicates that improvements could be made to the cell at the manufacturing

stage in an effort to improve its sensitivity if indeed a mirror-like surface is required to achieve this. The diamond polish recommended herein permits the re-attainment of the original sensitivity of the cell (as established by the manufacturing process).

The environmental recommendations made herein are more complete than have previously been described [e.g., Plotsky et al., 1978; Hjemdahl, 1984a] and were based on the observations that the ECD signal was most stable when the laboratory was at a temperature of 21 to 23°C with a humidity level between 30 and 60%. Similarly, the ECD output signal was more stable when the room was lit with incandescent lighting as opposed to fluorescent. The use of an uninterruptable power supply is recommended because the ECD is very sensitive to power line fluctuations when operating at 1 nA F.S.D.; the fluctuations are manifested as large deflections of the recorder pens and disrupted chromatograms.

The current choice of a reverse-phase phenyl column is unique for this analysis. As mentioned earlier (in Methods), the use of this column had been recommended for the separation of CA but had not been investigated. Instead, C_{18} columns are widely used in reported HPLC-ECD assays. The phenyl column functioned well giving an excellent resolution of the CA. However, the manufacturer (Waters Chromatography Division) of this phenyl column has, more recently, encountered difficulties in supplying stable, functional stationary phase. In fact, because suitable replacement

phenyl columns became unavailable, certain experiments planned above could not be fully completed.

(b) Chemicals Used in the Assay

The emphasis on the use of high-purity reagents, solvents and chemical products stressed herein is also unique in its' extent. [These data have not been presented in detail because they are too voluminous.] Once again, the necessity for these measures becomes very obvious at 1 nA. Contaminants and/or impurities can interfere with the CA themselves (e.g., by chelation), with the integrity of the chromatographic bed and with the functioning of the WE. These interferences can be manifested as poor recovery of the CA from plasma, poor resolution of the CA on the chromatograms, peak-splitting or tailing, diminished sensitivity of the ECD and/or increased baseline noise. These effects can occur immediately or can be delayed somewhat as cumulative effects and be either transient or persistent.

The commercial availability of reliable HPLC-grade chemicals, reagents and solvents has continued to improve so that, at present (1988), most chemical products do not need further purification in the laboratory (e.g., as had to be performed on the octanol above).

(c) Care of Associated Equipment

The specific need for the very clean glassware used in this assay is not emphasized in the literature. And while this may seem to be an obvious point, the use of non-residue detergents and double-distilled water for cleaning laboratory

glassware is not a universal practice. In light of the information given above, the avoidance of any source of extraneous chemical contaminants would seem to be prudent.

Similarly, the need to avoid losses of CA to the surface of glass containers seems self-evident. However, the siliconization of the glassware used in CA assays is rarely mentioned in the literature.

(d) Composition of the Mobile Phase

As far as the composition of the MP is concerned, the above recipes are unusual in that they contain a relatively high concentration of HSA. The use of the phenyl column has necessitated this to some extent and I have also attempted to capitalize upon the fact that HSA is a detergent and can purge (on a tonic basis) the column and detector cell. HSA would be expected to flush out any fatty acids originating in plasma and deposited within the system. Fatty acids were identified in the plasma extracts, and another source of fatty acids would be fingerprints. The latter are commonly deposited on the components of the apparatus during repairs to the system and even while the system is being used. The identification of the fatty acids in plasma as a potential source of contamination of both the column and glassy carbon electrode was novel, but this contamination can be readily avoided and/or eliminated.

The use of a 0.22 μm membrane filter for pre-treatment of the mobile phase was unique until recently [e.g., Foti et al., 1987]. While this is a rather obvious way of

ridding the MP of small particulate contaminants, the general practice was, and remains, to use a 0.45 μm membrane filter.

In summary, the success of all of these measures is clear from the low level of baseline noise that was normally obtained for the present assay, as demonstrated in Figure 21.

2. Extraction of CA from Plasma

The boric acid extraction technique of Smedes et al. appeared in the literature in 1982 but in spite of the apparent significance of this method, it has still not become widely utilized. This extraction technique was reported to be dependable and highly reproducible and to have a high efficiency. In contrast, the much more commonly utilized alumina extraction (Anton & Sayre, 1962) technique was known to be capricious, inefficient (typical recovery of CA is in the 40 to 60% region) and difficult to reproduce. The success of any particular use of the alumina as an extraction medium was highly dependent on the quality of the batch of alumina used. This, in turn, created a high degree of dependence on (unknown) manufacturing practices. Furthermore, the actual basis for the interaction of the alumina suspension with the CA has not been elucidated and consequently, has been outside of the control of the investigator. One of the reasons for the latter is that the science of alumina has changed drastically in the intervening years (i.e., since 1962) and while a great deal has been learned in the interim, this knowledge has not been transfered to the arena of CA analysis.

Commercially supplied "alumina" is actually a mixture of γ-aluminas. As opposed to α-alumina (corundum), which is pure Al_2O_3, γ-alumina is actually composed of six different compounds (namely: κ, η, χ, δ, γ, and θ). These are called the transition aluminas and have one of the following chemical structures: $Al(OH)_3$, $Al_2O_3.3H_2O$, AlOOH or $AL_2O_3.H_2O$. All of these compounds are catalytically active, in contrast to the α-alumina which is inert. [For a more detailed discussion of the physicochemical properties of γ-alumina, the reader is referred to Misra, 1986.] The surface of the γ-alumina powder (or suspension) has a range of Lewis Acid (and Lewis Base) and Brönsted Acid catalytic sites. Additionally, there is a pore size distribution on this surface which can accommodate molecules of varying sizes and shapes [Peri, 1965]. The alumina is thought to attract the organic nitrogen on the sidearm of the CA molecules [Fiedorow, 1988, personal communication]. Obviously, there is a large number of compounds present in plasma having an accessible nitrogen atom. This, then, may be the explanation for the lack of specificity of the alumina extraction technique as it applies to the extraction of CA from plasma.

Additionally, the alumina normally purchased for use in CA extractions (e.g., from Woelm Pharma, W. Germany) has had its Lewis Acid sites "poisoned" by the manufacturer by the use of either water or alkali ions. However, the manufacturer does not divulge the exact extent of this poisoning, saying only that "the degree of activity of the alumina is _____".

It is not possible, then, to know the actual surface characteristics of any particular batch of alumina. Therefore, different preparatory methods may account for the variability encountered between batches of alumina and extensively reported in the CA assay literature [Fiedorow, 1988, personal communication]. This also explains the basis for the "folklore" associated with the recommendations from different laboratories for "optimal" treatment of the alumina, and accounts for the uniqueness of individual batches of alumina. The above information on the chemical and physical properties of alumina has not been reported or accounted for in the CA literature.

In light of this information, the use of alumina as an extraction medium for CA seems contraindicated. The only exception to this rule would be in the event of an agreement between the "CA research community" and the manufacturers of the alumina for the preparation of a suitable and effective product. However, what exactly constitutes an "effective preparation" for CA extractions is unknown at this point in time.

For reasons including the above, the use of the boric acid extraction method of Smedes et al. [1982] was selected as the preferred method of extracting CA from human plasma for this study. The method is highly reproducible, reliable and efficient. The only changes made to the techniques outlined in the original paper were the use of ultrapure reagents and compounds (defined as having a level of contamination lower

than 10 ppm) as the consituents of the extraction solutions and the use of DOE as the internal standard instead of DHBA. This latter stipulation was necessitated by the current choice of column and chromatography conditions.

(3) Maintenance of Optimal Operating Conditions

Having established that it would be desirable to operate the HPLC-ECD system at a sensitivity level of 1 nA F.S.D. of the ECD on a continuous basis, a means of not only attaining this goal but also of maintaining it had to be found. Ideally, maintenance and quality control procedures should not interfere with the routine functioning of the apparatus (i.e., minimal "down-time"). And if these procedures must disrupt the functioning of the apparatus, then the techniques employed should at least be able to completely restore the level of function lost due to contamination of the system with use. Logically, these techniques should be employed on a regular basis so as to ensure that the HPLC-ECD system runs optimally on a chronic basis. [For a more detailed discussion of the principles of maintenance of assays in a laboratory, the reader is referred to Stewart & Koepke, 1987.]

In the present instance, a number of strategies were evolved by which the HPLC-ECD apparatus could be maintained contaminant-free and/or cleaned. These were extensively described in Methods, Section A(4). The overnight MPs that were developed and described here are unique. These solutions proved to be very effective in maintaining a low level of

baseline noise and in preserving the sensitivity of the ECD cell (i.e., delaying passivation of the electrode surface) with minimal "down time".

In the present instance, a 25% diminution of maximum attainable signal response of the ECD to the CA was chosen as the point at which the system was cleaned by more aggressive techniques than the overnight MPs and the methanol injections. It is important to appreciate that the HPLC system and the ECD detector require different and individual approaches to cleaning and that a way of cleaning both of them simultaneously and compatibly must be found. Instructions for cleaning an HPLC-ECD system are usually a part of the operation manuals supplied by the manufacturers of the individual components. The recommendations made include a methanol wash (safe for the entire system), a nitric acid rinse (safe only for the pump and injector and the ECD cell on occasion), and the scrubbing of the active surface of the WE with "alumina" (usually supplied by the manufacturer of the ECD).

During vigorous cleaning of the HPLC-ECD system, the ECD cell is turned off and the reference electrode is placed in storage. Whenever the ECD cell has to be turned off for a period of time greater than a few minutes, a period of re-equilibration is necessary before the cell is usable again. The higher the desired operating sensitivity, the longer will be the re-equilibration time. When this assay was first established, the re-equilibration period that followed a

methanol wash of the whole system was 7 to 10 days. After the above modifications were made to the equipment and methods, this re-equilibration period was reduced to approximately 48 hours. This was one of the most dramatic examples of the effectiveness of the electrical grounding, upgrades of the ECD and the above variety of stabilization techniques employed by the present assay. The issue of the minimization of "down time" has not previously been fully addressed in the HPLC-ECD CA assay literature.

As described on several occasions in this text, the polishing of the WE of the ECD to restore its sensitivity has been the subject of some controversy [Anton, 1984]. Previously, the recommended polishing medium for the glassy carbon WE was a suspension of alumina (e.g., see BAS LC-4B Operator's Manual). The assumption was made, but not specified, that this alumina be composed of α-alumina (i.e., corundum). Furthermore, if this proved ineffective in restoring the sensitivity of the cell, it was recommended that a new cell be purchased.

As described earlier, any type of alumina, other than α-alumina, would interact with the CA and possibly interfere with their oxidation. The photomicrographs of the surface of the glassy carbon electrode demonstrated that alumina particles had persisted in the grooves of the lines running across the WE in spite of sonication. And the XRD studies verified that the alumina suspension is indeed a mixture of

α- and γ-aluminas. In addition to the risk of a catalytic effect of this mixture of aluminas on the CA, effective polishing of the electrode cannot be achieved by the use of this compound: corundum, with a hardness of 9 on the Mohs scale, is capable of polishing the hard surface of the glassy carbon electrode but mixed aluminas were much less effective.

The chromic acid treatment of glassy carbon electrodes described by Anton (1984) is a rather drastic measure that cannot be used on a regular basis without risk of inflicting permanent damage to the electrode. This is also true of the previously used nitric acid washes.

The use of the diamond suspension as the polishing method for restoring the glassy carbon electrode to its original sensitivity is unique. The proof of the effectiveness of this medium lies in the peak heights achieved with the standard CA solutions and recorded on both the polarograms and the standard curves (Figures 10 and 15 to 18). These data were all collected after polishing the WE with the diamond suspension. Therefore, the diamond polishing technique described is a practical and effective method of restoring the sensitivity of the glassy carbon WE.

Recently, a letter was published in Clinical Chemistry describing the loss of sensitivity of the ECD cell over a period of time [Masse et al., 1988] confirming the observations presented above. However, the authors merely report the phenomenon of the diminishing sensitivity of the ECD cell without recommending a solution to the problem.

The use of the Shimadzu integrator and the 1 nA setting on the ECD facilitated the identification and resolution of this problem by the presented method some time ago. Once again, CA assays most commonly reported in the literature describe the function of the ECD at 5 nA, or, at best, 2 nA F.S.D. [see, for example, Foti et al., 1987]. At these sensitivity ranges, modest diminutions in maximum obtainable response or sensitivity are not readily apparent and, of course, the usefulness of these assays for low concentrations of CA is compromised.

4. Validation of Assay Technique

The fact that all of the validation experiments performed in support of this assay were carried out at 1 nA F.S.D. is unique but the results obtained are quite typical for an HPLC-ECD CA assay [e.g., Davies & Molyneux, 1982]. The derivation of the polarogram at 5 nA F.S.D. with only 1000 pg of each CA is unique (as is the extent of the response of the ECD) but the patterns obtained for the CA are typical [e.g., Causon et al., 1981]. The variabilities observed for the repeat extractions of plasma and injections of standards were very small, contributing insignificant error to the assay method. Thus the limitations of the present assay are related to the apparatus and the details of its operation.

What is unique in this assay is the achieved level of attention to such details in spite of earlier emphasis to do so [Hjemdahl 1984a; Weicker et al., 1984]. The resultant

success of the measures employed has overcome a number of long-standing problems with HPLC-ECD CA assays. However, while the present method requires conscientious attention to the fastidious operation of the HPLC-ECD system as described, it is limited by the current state of development of the hardware used.

Nonetheless, the present assay contributes advances to this area of study. In a recent paper, Nyyssönen & Parvianen (1987) identified a number of "sources of error" with HPLC-ECD CA assays but had few solutions for them. /These authors advocate the use of alumina yet complain about low recovery of CA, admit to producing chromatograms which have a NA peak starting on the tail end of the solvent front, complain about inescapably long retention times and refer to papers which had been published six years previously describing similar problems with the HPLC-ECD CA assay. The authors go on to state that the amperometric ECD is "unstable" but do not offer suggestions as to how to diminish this instability.

The above paper is pertinent because it illustrates the extent of the enduring frustrations and problems with HPLC-ECD CA assays. Furthermore, it provides an indication of the longevity of the problems associated with this assay. The authors of this paper refer to their own assay as an example of a workable CA assay protocol but their assay method is published in Finnish, in a Finnish journal, and thus is not readily accessible for review. Other recently published methods for CA assays are no more helpful.

As discussed above, any HPLC-ECD assay, to be useful, must be paired with an efficient plasma extraction method. Secondly, the HPLC-ECD must be demonstrated to be capable of functioning at 1 nA F.S.D. for reasonable periods of time. Recently published CA assays employing HPLC-ECD have not met these criteria and, hence, offer no real gains over the assays which previously were proven to be inaccurate [Hjemdahl, 1984b]. Examples of some such assays are mentioned below.

In an effort to take advantage of HPLC technology and the sensitivity of the REA, Feoli et al. (1988) have presented a CA assay that combines radioenzymatic conversion of the CA to their more stable metabolites, extraction of the radio-labelled compounds with boric acid-organic solvent extraction followed by HPLC separation and ECD detection. Part of the expressed rationale for the use of boric acid was that the authors were trying to avoid the inhibitory effects of alumina on COMT. No chromatograms were shown in this publication and the method described is obviously very labour-intensive. Little evidence was presented that the assay does not harbour many of the problems of both the REA and HPLC-ECD methods.

In 1985, MacDonald & Lake reported an HPLC-ECD CA assay with an extraction method which employed boric acid-organic solvent extraction of CA but preceded by alumina precipitation of the plasma. Based on the information above, anything that could have been gained (in terms of recovery of CA) with boric acid had already been lost by pre-treatment of the plasma with alumina.

Another, recent publication describing an HPLC-ECD CA assay [Foti et al., 1987] does not contain any polarograms, uses alumina extraction of CA from plasma, reports recoveries of CA from plasma in the 60% range (at a low concentration range), and makes no mention of how the HPLC-ECD apparatus is maintained. The published chromatograms were recorded at 2 nA F.S.D. on the ECD and an injection of CA standards produced a chromatogram having a NA peak with interference from the tail end of the solvent front.

The use of boric acid gels continues to be reported in conjunction with HPLC-ECD CA assays [Imai et al., 1988] but this extraction method has also not received a wide following. The attractiveness of boric acid gels for the automation of CA assays cannot be realized before the resolution of the variety of the above-mentioned HPLC-ECD problems. These authors present their chromatograms recorded on a strip chart recorder with the range setting on the ECD adjusted in mid-chromatogram (from 2 nA to 4 nA F.S.D.) to accommodate late eluting peaks that would otherwise have been off-scale on the strip chart recorder.

In summary, the problems associated with operation of the HPLC-ECD apparatus for the purposes of CA assays and the question of complete extraction of CA from plasma have endured in the literature.

The HPLC-ECD apparatus is still evolving as new technology is developed and becomes commercially available. Improvements continue to be made to the support technology of

this assay (e.g., the availability of ultra-pure, "HPLC-grade" reagents and chemicals). However, there is still considerable basic research to be done on the apparatus and on the systems as a whole. For example, the actual physical-chemical properties of the glassy carbon, thin-layer electrodes have not yet been fully characterized and improved ECDs may be expected to evolve.

In summary, an effective method of extracting CA from plasma has been developed (Smedes et al, 1982) and while it presents significant advantages over previous techniques (e.g., Anton & Sayre, 1962), its specificity is still imperfect.

However, methods are presented herein by which a currently available HPLC-ECD system can be maintained at a high level of sensitivity (namely, 1 nA F.S.D. on the ECD) on a continual basis. The meticulous use of these methods has been shown to be effective in providing accurate CA assays of plasma. While this assay cannot yet become a routine procedure in a clinical laboratory, it has been developed to a level similar to other special analyses (e.g., estrogen receptor assays of breast cancer) performed in dedicated laboratories by specifically trained staff.

B. Patient Studies

1. SAH Study

The experience with the exercise study reported in Appendix I revealed the need to allow each individual to act

as his/her own control in CA studies. Secondly, the variability observed for the magnitude of "normal" CA response was noteworthy. Therefore, a study protocol was designed in such a way as to accommodate the needs of responsible and appropriate collection of plasma in a clinical setting; the example chosen was a study of plasma CA levels during the clinical progress of patients who had suffered an SAH due to rupture of a cerebral artery aneurysm. This study proved to be an enormous challenge on a daily basis because of the restrictions imposed by the method of blood collection.

Experience with collecting the blood samples on Unit 4A4, in general, re-affirmed that the choice of sampling times was appropriate (06:30 and 16:30) as was the number of samples to be collected (twice per day). These sampling times had been chosen in an effort to avoid both the peaks in the circadian rhythm of plasma CA levels [Ratge et al., 1982] as well as the busiest time of day in the Intensive Care Unit, insofar as that was possible. The morning sample was most appropriate for this purpose but the afternoon sample was planned in the event that something went awry with the morning sample, as happened on occasion.

A number of problems culminating in an inability to obtain blood samples were encountered. For example, blood samples were unobtainable if a patient was being transfused, if the patient had been transferred to the radiology department for a computed tomography (CT) scan, if the patient was eating, or if the patient had visitors and was thus in an

aroused state. It often happened that the i.v. lines were non-functioning (either occluded or mis-positioned) and had to be re-inserted; if the patient was due for some other procedure immediately thereafter, it was not possible to allow sufficient recovery time prior to sampling. Alternatively, the patient might not have a vein suitable for sampling. Not infrequently, patients were being stimulated by chest physiotherapy or other manoeuvres and since these were expected to affect the plasma CA concentration, blood collection had to be delayed. Often these problems came in multiples and even though much effort was expended in overcoming these obstacles (i.e., re-starting i.v.'s, waiting for the patient to settle down after various therapeutic procedures, returning at odd hours to collect blood and so on), samples were occasionally missed or could not be salvaged.

In addition to the problems encountered in obtaining blood samples, there were also difficulties associated with obtaining information on physiological parameters (i.e., heart rate and blood pressure) at the time of the blood sample. It was often not possible to take these measurements immediately after the blood sample because of the complexity of the functioning of the Unit. Therefore, many of the HRs and BPs reported for the patients in this study were not immediately associated with the sampling times and thus this information cannot be correlated with their plasma CA levels.

The extent of the variety of the drugs administered to these patients was impressive and could not have been anticipated. The drugs given to these patients included: Valium, morphine, acetominophen, Gravol, codeine, pitressin, atropine, Dalmane, Metoprolol, Diazide, Corgard, Minipres, Cloxacillin, Decadron, digoxin, Xylocaine, Ventolin, heparin, Pavulon, Lasix, Dilantin, Tempra and DA (by infusion). This list does not include drugs given during surgery.

The continuing assistance of the attending medical and nursing staff of the Unit cannot be over-emphasized in a study such as this. Without their cooperation and vigilance, this type of study could not have been performed in an acute care setting. The practical implications of this study were novel to the staff and if a study such as this were to be repeated at this hospital, it would probably proceed more smoothly. Problems including those encountered with obtaining blood samples, providing education and counselling to the families of patients (related or unrelated to obtaining consent) and having conferences with the staff often took well over 6 hours of the day. This would be an excessive commitment of time if the assay were also to be kept functional on a routine basis by a single individual.

Ultimately, 14 patients met the criteria for inclusion in the CA study. The results of only five of these patients were reported above. This latter group of patients all had a GCS of 13 to 15 at admission and only one of them (PN-21) went on to develop vasospasm. With the exception of PN-21, their

clinical courses were uneventful. They all exhibited a single spike in the CA profile during the course of their participation in the CA study but the elevations noted were all within the reported range of normal in the literature. Some of these increases could be attributed to an artefact caused by the drugs that had been administered (i.e., the medication interfered with the resolution of the CA on the chromatogram) while others correlated with a lack of sleep and a bad headache.

It is interesting that it was the concentration of A which most often rose to several times the admission levels while NA was relatively unaffected. It is possible, that the elevation of A was an artefact caused by drug peaks superimposed on the A peak. All of the chromatograms produced exhibited numerous spurious peaks. As discussed above, A is harder to measure than NA in that it is less electroactive and less well extracted from plasma than NA. Any error in peak height measurement caused by drug interference would, therefore, result in inaccurate A quantification. An increase in plasma A levels has been documented in the literature as a result of mental stress [Eliasson, 1984]. However, the significance of this fact in this group of patients is unknown.

The other nine patients admitted to the CA study were largely poor grade SAH patients (GCS less than 10). Their plasma samples could not be analyzed for CA content due to the apparent overwhelming presence of drugs and drug metabolites.

The boric acid extraction technique probably could not differentiate between drugs (and their metabolites) targetted specifically for the SNS and the native CA, so many of these compounds were extracted from the plasma simultaneously. (Many anesthetics and analgesics have a molecular structure similar to that of the CA.) Additionally, patients were given infusions of DA to raise their blood pressure if it fell below the acceptable range (acceptable from the point of view of maintaining adequate perfusion of the brain). This confounded both the endogenous levels of CA and the chromatograms.

The effects of the drugs on the assay were many and varied and included permanent deposition on the column and WE and interference with the resolution of the CA on the chromatograms. The range and extent of these effects was unpredictable. The interference of drugs with CA peaks on the chromatogram was the subject of a recent paper [Koller, 1988]; however, the assay method reported used alumina extraction of the spiked plasma and the chromatography conditions were different from those reported herein. The possibility of such interference by drugs had not been raised in the SAH literature dealing with CA studies of patients. In fact, there is sparse discussion of these factors in the literature as they pertain to CA studies, with the exception of certain cardiac drugs which affect chromatograms [Bouloux & Perrett, 1985].

It was of interest to be able to compare two sisters who had had different treatment regimens. According to previous

experience with this group of patients, the timing of surgery (i.e., immediate vs. delayed) does not have an adverse effect on patients having small hemorrhages and no neurological deficits [Kassell et al., 1981]. Both sisters had small hemorrhages (i.e., low volume of blood released into the subarachnoid space), maintained a good GCS, had good blood pressure regulation and low plasma CA concentrations throughout the time course of the study protocol used. Therefore, the sisters verified the general experience that the prognosis is good for patients with small bleeds and good GCS throughout.

With the small sample size, it is not possible to draw conclusions about a relationship between plasma CA concentrations and the development of vasospasm based on this study. Even if all the patients studied had been included, many were receiving DA (i.v.). What can be suggested is that good-grade patients with small bleeds (these patients already have a good prognosis based on the extent of their hemorrhages alone [Wilkins, 1986; Drake, 1981]), and without highly elevated CA, did well.

2. Comparison With Other CA Studies of SAH Patients

The problem of studying the role of CA in the etiology of cerebral artery vasospasm can best be illustrated by a comparison of two recent review articles on this subject, those of MacKenzie & Scatton (1987) and De Salles (1987). In the latter review paper, the discussion interchangeably

considers the development of vasospasm in humans and in animal models; experimental and spontaneous intra-cerebral hemorrhage are lumped together; the issue of the integrity of the blood-brain barrier is generally ignored; the influence of drugs on any or all of the above aspects are ignored and the role of Ca^{++} is summarized in one brief paragraph. In contrast to this, MacKenzie and Scatton delve into each of these topics in detail and discuss the implications of the interactions between various factors thought to be relevant to vasospasm. These latter authors provide a more realistic appraisal of the intricacies associated with the study of SAH and delineate at least some of the issues which must be confronted in circumspect investigations.

The studies in the literature describing attempts to investigate the involvement of the CA in the development of complications following SAH [e.g., Yoshida et al., 1987; Benedict & Loach, 1978; Loach & Benedict, 1980] have not included consideration of the special demands of CA research conducted on intact humans. In none of these studies was there an attempt to obtain plasma in a uniform or systematic fashion. For example, in the 1980 study by Loach & Benedict, plasma samples were obtained at 6-hour intervals for 1 day post-operatively and then only once again at the time of discharge. This and other studies have also suffered from a number of other short-comings including the sampling of plasma at random times during the stay in hospital, sampling at random times of day, collection of a random number of samples,

undisclosed methods of plasma sampling, no consideration of drug administration, no revelation of the patients' postural position during the blood sampling, no record of the patients' heart rates and blood pressures at the time of the sampling, and no general information about the age or general state of health of the patients (including such important matters as their cardiac condition) [e.g., Elworthy & Hitchcock, 1986; Minegishi et al., 1987a; 1987b].

C. Concluding Remarks

A reliable and reproducible human plasma CA assay using the technique of boric acid-organic solvent extraction coupled with HPLC-ECD has been presented. The assay is capable of accurately quantifying CA in drug-free plasma. However, the assay method continues to be limited by available technology and by the manufacturing performance of the companies providing the materials. It is recommended that as advancements are made in the technology, they be incorporated into the assay and that vigilance is maintained in monitoring the quality of commercially acquired components and materials.

The study of the role of CA in the development of vasospasm post-SAH was too limited to permit conclusions to be drawn about underlying physiological mechanisms. However, it did succeed in identifying more clearly the short-comings and pitfalls of attempting to perform CA studies in an acute care setting (i.e., in a Neurosurgical Intensive Care Unit). The

information gleaned can be used to design other studies that would have a higher probability of yielding new information on the changes in plasma CA levels following a SAH. In light of the severity of the ravages of vasospasm, the elucidation of the contribution of CA to exacerbating this complication of SAH is worth pursuing.

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APPENDIX I

Reproducibility of the Catecholamine Response to Maximal (Dynamic) Exercise

By 1980, it had been well established that the physiological responses of an individual to an exercise stimulus are highly reproducible [Åstrand & Rodahl, 1977]. That is, approximately identical heart rate (HR), blood pressure (BP) and oxygen consumption ($\dot{V}_{O_2}$) will be seen from one day to the next in response to the same pattern of exercise stress, such as during a progressive bicycle ergometer test. Yet the reproducibility of certain humoral responses to this same stimulus were not necessarily as well understood nor as predictable. While it was known, for example, that the concentration of plasma catecholamine(s) (CA) [namely adrenaline (A) and noradrenaline (NA)] increase in response to exercise, the nature and reproducibility of that response was not known [Cronan & Howley, 1974]. The purpose of this study was to determine the reproducibility of the CA response (as revealed in peripheral venous blood) to a repeated dynamic exercise stimulus.

The plan of the study was as follows:

Eleven young men volunteered for the study. Their mean age was 21.9 years; their average height and weight were 1.79 m and 76.2 kg, respectively.

On day 0, the subjects underwent a preliminary maximal bicycle ergometer test to familiarize them with the exercise

laboratory and to provide an estimate of their maximal exercise capacity. Their HR, BP and $\dot{V}_{O_2}$ were monitored continuously throughout the maximal bicycle ergometer test but no blood was drawn for CA analysis. On day 7, they returned to the laboratory for their first experimental test with blood drawn for CA analyses; the same test using exactly the same protocol was subsequently repeated on day 14. The protocol for these tests is illustrated in Figure 42.

All tests were performed on an electrically braked bicycle ergometer with continuous measurement of HR, BP and oxygen consumption. Upon arrival in the laboratory, the subject had an indwelling cannula inserted into an antecubital vein. Following ten minutes of rest, a sample of blood was collected for baseline determination of CA. Ten minutes later, at time 0, the test was started with an initial load of 500 kpm (kilopond meters/min) for 1 minute with increments of 300 kpm every three minutes thereafter. A subject's effort was judged to be maximal when he could not maintain 3 minutes at a particular workload. Blood was sampled for CA analysis at three minute intervals throughout the test.

The time points of greatest interest to the reproducibility study were rest, 60% of maximum effort, maximum effort, 30 seconds after the end of the test and at recovery (approximately 15 minutes post-exercise). The plasma samples collected on these occassions were handled essentially as described in Methods (above) and were assayed in duplicate (and at random) according to the radioenzymatic (REA) method

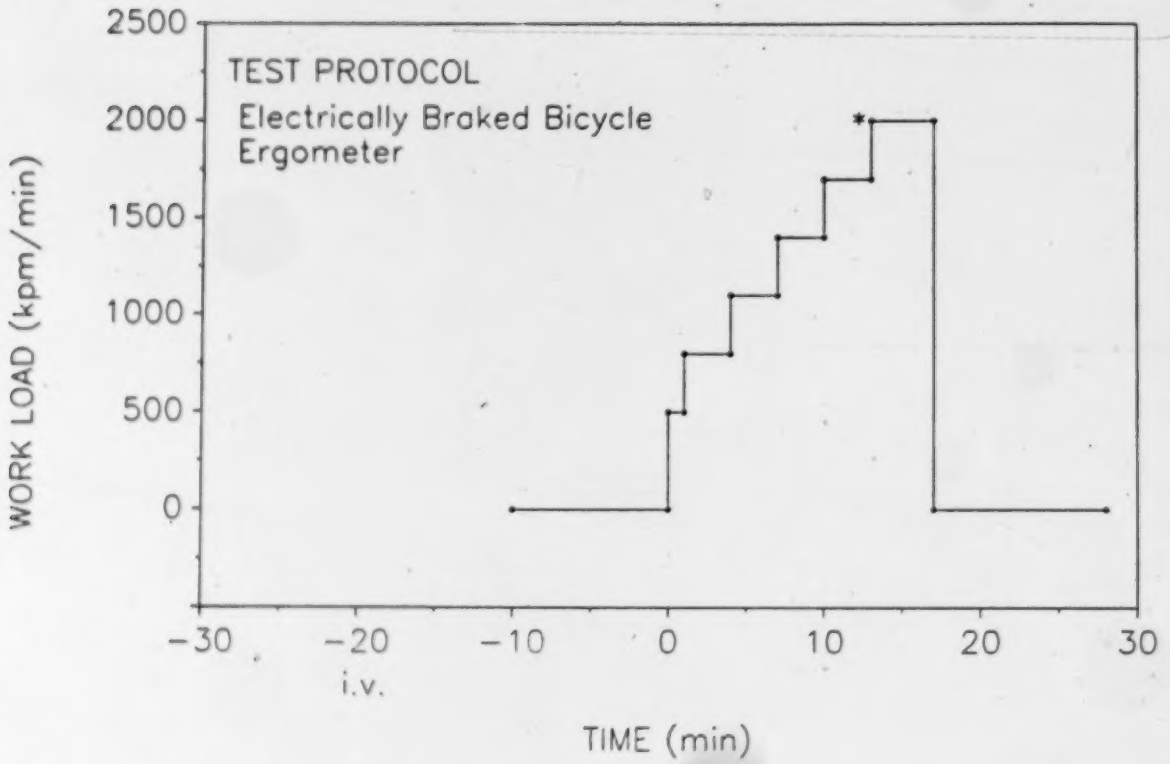


Figure 42. Test protocol for determination of the reproducibility of the plasma catecholamine response to dynamic exercise. * indicates point of maximal effort.

of Hörtnagl et al. (1977) without modification. The results from one subject (MO) will be discussed as an illustration of the results obtained from the group [See Figures 43 & 44].

It is obvious from the figures that the physiological parameters behaved predictably and in a highly reproducible fashion: the end points were, in every case for HR, BP and $\dot{V}_{O_2}$ very nearly identical between the two tests. In contrast, for each CA, the results varied widely from the first to the second test and this was particularly evident at the maximal points. (It is important to remember that the physiological parameters were virtually identical at this same point in time.) Additionally, while both A and NA increased in response to exercise, the magnitude of the increase in the concentration of NA was much greater than the magnitude of the increase in the concentration of A. The 30-seconds post-exercise sample was also of interest: As expected, the physiological variables (e.g., heart rate) began to fall rapidly in both tests right after the end of the tests. In contrast to this, however, the NA concentration in plasma started to fall after Test 1 but continued to climb after the end of Test 2.

In all eleven subjects, this was the overall pattern observed. That is, the physiological parameters varied predictably in response to the increments in workloads. While the concentration of CA in plasma increased in response to the exercise, the magnitude of that increase was unpredictable; in some subjects, the first test yielded the highest plasma CA

Figure 43. Plasma catecholamine concentrations in response to two consecutive exercise tests. Data are from one subject. * indicates point of maximal effort.

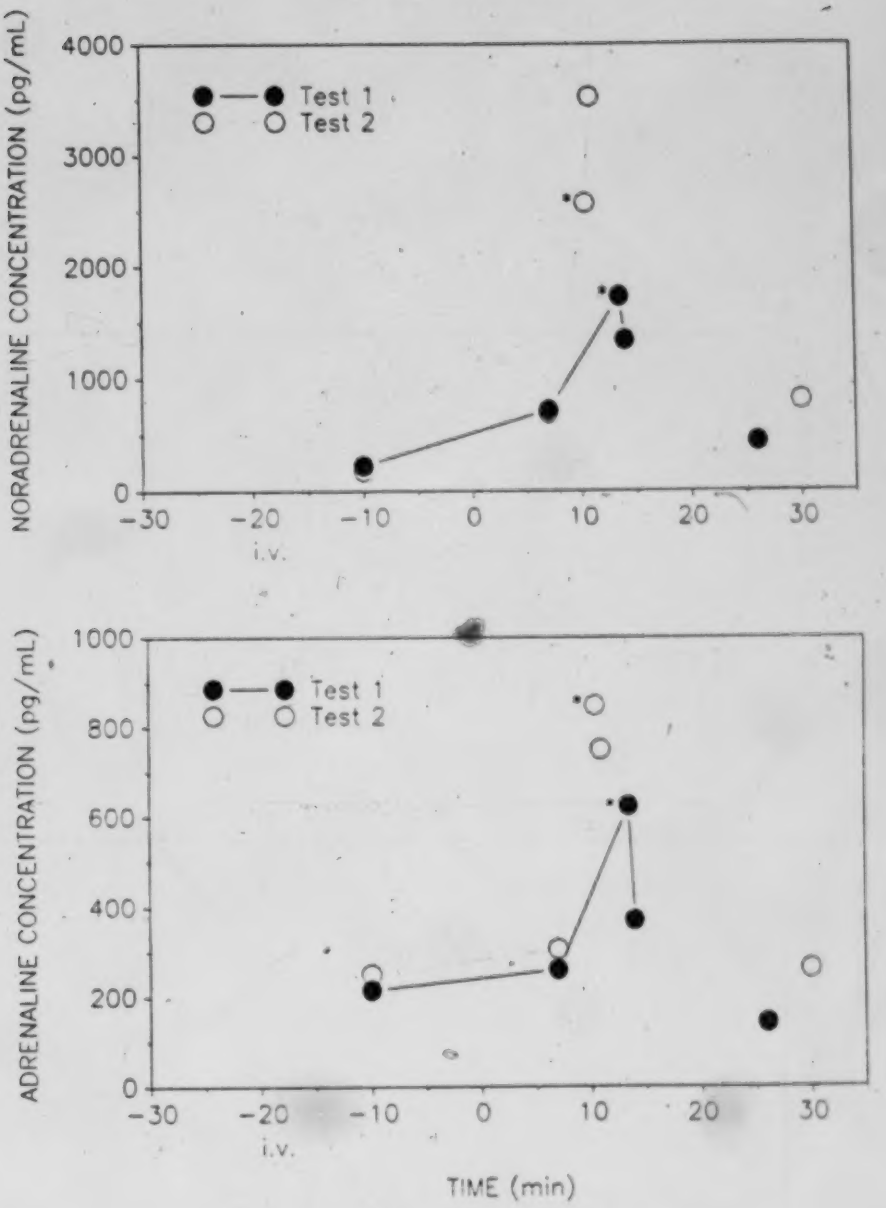
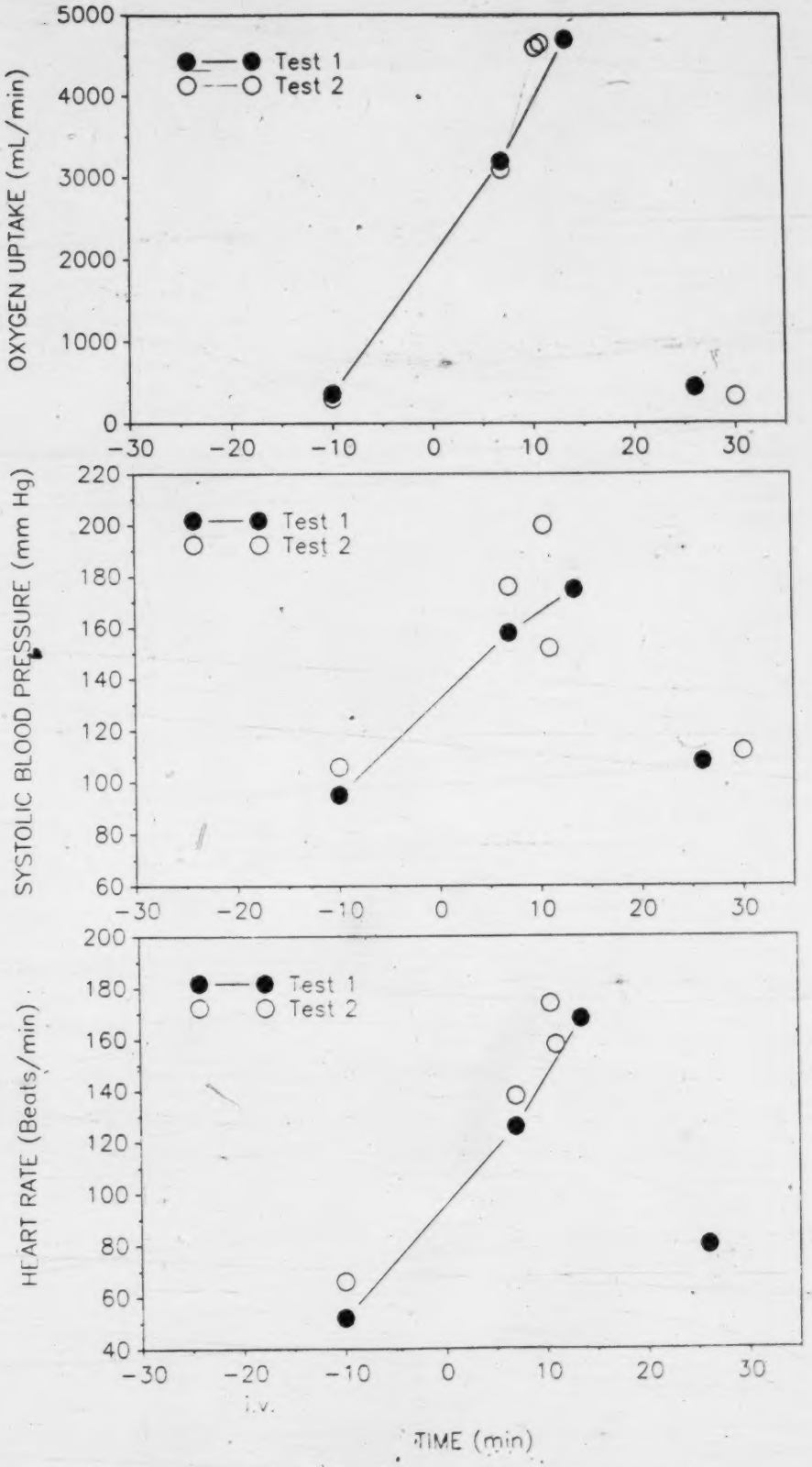

NORADRENALINE CONCENTRATION (pg/mL)
4000
3000
2000
1000
0
Test 1
Test 2
−30
−20
−10
0
10
20
30
i.v.
ADRENALINE CONCENTRATION (pg/mL)
1000
800
600
400
200
0
Test 1
Test 2
−30
−20
−10
0
10
20
30
i.v.
TIME (min)

Figure 44. Physiological responses to two consecutive exercise tests (see Figure 43).


OXYGEN UPTAKE (mL/min)
5000
4000
3000
2000
1000
0
Test 1
Test 2
−30
−20
−10
0
10
20
30
SYSTOLIC BLOOD PRESSURE (mm Hg)
220
200
180
160
140
120
100
80
60
Test 1
Test 2
HEART RATE (Beats/min)
200
180
160
140
120
100
80
60
40
Test 1
Test 2
i.v.
TIME (min)

values while in other subjects, it was the second test that yielded the higher values. In an attempt to summarize this divergence, the difference between the values obtained in the two tests for all subjects at the submaximal and maximal workloads was averaged [See Table 9]. That is, the differences in HR, BP, $\dot{V}_{O_2}$, and the concentrations of A and NA at the two workloads in both tests were tabulated. [The actual data obtained at each of these time points is also given, as a point of reference, on the same Table. The raw data is presented in Table 10.]

The variation in HR between the two tests was small (<3%) as were the BP and oxygen consumption. However, the variability of the concentrations of A and NA was very large (i.e., the standard errors of the mean were as much as approximately 50% of the means). For example, the actual mean value obtained for A at the submaximal and maximal workloads (in test 1, for example) for ten subjects was 228 and 701 pg/mL plasma, respectively. Yet the A varied by an average of 98 pg/mL and 348 pg/mL at the submaximal and maximal workloads, respectively. The same was true for NA: at the submaximal workload, the mean difference noted was 248 pg/mL while the actual mean value was 635 pg/mL (also test 1); and at the maximal workload, the actual mean value was 2662 pg/mL (test 1) while the mean difference between the two tests was 1024 pg/mL.

The most obvious explanation for this variablity was the question of the degree of error inherent in the CA assay

Table 9. Comparison of physiological and catecholamine responses to exercise in two consecutive tests of human volunteers.

Effort	Heart Rate (Beats/min)		Oxygen Uptake (mL/min)		Plasma Catecholamine Concentrations: Adrenaline (pg/mL)		Plasma Catecholamine Concentrations: Noradrenaline (pg/mL)	
	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
Submaximal	137 ± 5	142 ± 5	3282 ± 94	3331 ± 130	228 ± 35*	171 ± 22	635 ± 75*	684 ± 70
Maximal	174 ± 2	176 ± 2	4950 ± 113	4947 ± 126	701 ± 78	679 ± 120	2662 ± 349	2531 ± 326
	Absolute Differences Between Test 1 and Test 2							
Submaximal	6.8 ± 1.5		156 ± 33		98 ± 41*		248 ± 64*	
Maximal	4.4 ± 1.3		206 ± 41		348 ± 72*		1024 ± 223	

Means ± S.E.M.; N = 11; Submaximal = 60% of maximal exercise effort.
* N = 10.

Table 10. Data for Test Results Summarized in Table 9.

A. Submaximal Effort

HR (beats/min)			$\dot{V}_{O_2}$ (mL/min)			ADR (pg/mL)			NORADR (pg/mL)		
Test 1	Test 2	Diff.	Test 1	Test 2	Diff.	Test 1	Test 2	Diff.	Test 1	Test 2	Diff.
150	156	+6	3050	2910	-140	159	238	+79	820	978	+158
132	145	+13	3650	4100	+450	188	168	-20	270	571	+301
126	138	+12	3200	3100	-100	260	306	+46	717	693	-24
108	123	+15	3100	3280	+180	92	207	+115	642	930	+288
138	135	-3	3450	3350	-100	149	153	+4	353	513	+160
132	134	+2	3100	3150	+50	248	128	-120	1130	511	-619
153	160	+7	3300	3200	-100	498	54	-444	558	1064	+506
146	147	+1	3300	3150	-150	209	114	-95	665	326	-339
160	168	+8	3950	4200	+250	266	219	-47	560	562	+2
146	138	-8	3200	3300	+100	212	204	-8	634	553	-81
118	118	0	2800	2900	+100	---	92	---	---	817	---
x ± S.E.M.:											
137±5	142±5	6.8±1.5	3282±94	3331±130	156±33	228±35	171±22	98±41	635±75	684±70	248±64

Table 10. Continued...

B. Maximal Effort

HR (beats/min)			$\dot{V}_{O_2}$ (mL/min)			ADR (pg/mL)			NORADR (pg/mL)		
Test 1	Test 2	Diff.	Test 1	Test 2	Diff.	Test 1	Test 2	Diff.	Test 1	Test 2	Diff.
180	180	0	4810	4890	+80	1199	608	-591	4686	4413	-273
172	175	+3	5300	5700	+400	687	989	+302	1761	2904	+1143
168	174	+6	4700	4600	-100	624	849	+225	1729	2569	+840
172	182	+10	5480	5320	-160	575	1465	+890	---	---	---
162	168	+6	4580	4800	+220	256	462	+206	1223	2361	+1138
174	174	0	4250	4400	+150	603	449	-154	3774	1972	-1772
177	180	+3	4700	4300	-400	868	229	-639	1725	1638	-87
183	181	-2	5200	5250	+50	450	464	+14	2252	2339	+87
180	180	0	5150	5060	-90	661	429	-232	3209	1718	-1491
176	180	+4	4980	5200	+220	786	1219	+433	2856	4114	+1258
170	156	-14	5300	4900	-400	996	309	-687	3433	1282	-2151
x ± S.E.M.:											
174±2	176±2	4.4±1.3	4950±113	4947±126	206±41	701±78	679±120	348±72	2662±349	2531±326	1024±223

technique. However, the linear regression equations for NA and A standards in the assay (in the concentration range, 750 to 3250 pg/mL plasma) were:

NA: $y = 1.012x + 1.41$; $r = 0.98$

A: $y = 1.067x - 0.98$; $r = 0.98$

This indicated that there was minimal, inherent systematic error in the assay. The coefficients of variation for measurement of A and NA were 2.1% and 3.8%, respectively, indicating minimal random error inherent in the performance of the radioenzymatic CA assay.

It was concluded that the variability in the CA measurements seen between the two tests was not an artefact of the assay technique and represented a true physiological difference in humoral responses between the two tests [Damjanovic & Kappagoda, 1981].

These results have been confirmed on several occasions since that time [e.g., Ward et al., 1983; Hyyppä et al., 1986]. These data are significant in that they indicate that it is imperative that individuals act as their own controls in CA studies; and, they argue for the fact that each subject participating in a CA study should be evaluated independently rather than as part of a group phenomenon [Holly & Makin, 1983]. Furthermore, these results suggest caution in the interpretation of data on the concentrations of plasma CA or the magnitude of change of plasma CA concentrations in clinical settings.

Appendix II

Summary of the Principles of Chromatography

Chromatography is a technique whereby the components of a mixture can be separated from one another by the exploitation of subtle differences between their physicochemical properties even if the molecules are very similar to one another. A chromatographic system is composed of at least two main parts: a mobile phase and a stationary phase. Typically, the mobile phase (MP) is a gas or a liquid carrying the mixture to be separated over an immobile surface, the stationary phase (SP). The components of the mixture exhibit differential degrees of interaction with the SP: some of them are attracted to the SP and retained by it; other analytes are not, and continue to flow through the system at essentially the same velocity as the MP [Section III, in Williams et al., 1978].

This difference in "transit time" is the basis for the separation of the components of the mixture by the column. It is worth noting that the actual properties which control the interaction between the MP and SP include absorption and adsorption, solubility, molecular shape and size, ionic charge and specific binding affinity. In this way, subtle separations become possible and a wide variety of applications becomes feasible [Tietz, 1986].

Chromatographic techniques can be classified in one of two ways: either on the basis of the interactions taking place (e.g., partition chromatography, steric exclusion

chromatography, etc.) or on the basis of the physical state of the mobile and stationary phases (e.g., gas-liquid, liquid-liquid, liquid-solid, etc., chromatography). This latter method is more common and has, in fact, been further simplified to designate only the type of stationary phase employed. For example, in the case of types of liquid chromatography (LC): a mixture of components is separated into its constituent compounds by flowing a liquid MP over a solid SP. The SP could be either paper, or a coated glass or plastic plate, or a tube ("column") packed with a bonded-silica material [the latter configuration effectively constitutes an immobilized liquid]. The name of the chromatography would thus be paper, thin layer and column liquid chromatography, respectively. Due to recent advances in the technology of columns, this last field has expanded so much that methods are now referred to by the type of column employed (e.g., reverse phase or ion-exchange chromatography).

The radioenzymatic assay (REA) that is often employed for human plasma CA measurements uses thin-layer chromatography (TLC), a type of liquid-solid chromatography. In TLC, silica gel (or some other adsorbent) is spread as a thin layer (e.g., 0.25 mm) over a glass or plastic plate (typically 20x20 cm size). These plates (SP) are usually scored along the vertical axis to create individual channels that do not overlap with each other. The mixture to be separated is spotted or streaked onto a channel near the bottom edge of the plate and allowed to dry. The plate is then placed inside a

closed glass tank which has a 1 cm deep layer of liquid MP (specially formulated for the separation to be effected) lying at the bottom of it. The MP migrates up the plate by capillary flow.

Each of the components within the sample will have a different degree of interaction with the silica/alumina particles (either associating with the particles and being detained by them or else being unattracted to them and passing them by). Riding on the flowstream of the MP, the sample thus becomes separated into zones of compounds. These migrating zones of individual components of the sample mixture are known as bands. The migration of a band through the column is known as the elution of a compound.

The separation of the compounds in the mixture is stopped by taking the plate out of the tank at the pre-determined time. The MP is allowed to evaporate from the plate and the compounds of interest are now separated from each other in individual bands which can either be visualized with the aid of dyes or ultraviolet light or be detected by radioactivity or chemical reactivity [Chapter 8 in: Williams et al., 1978]. The distance of a band from the origin of the plate is characteristic for each compound and is the basis for their identification. If further purification or quantification is desired, the silica gel can be scraped off the plate (in individual bands), the compounds lifted from the silica and subjected to further reactions.

It is important to appreciate that chromatographic systems can only separate finite numbers of molecules in a mixture before they become overloaded. Therefore, chromatography is usually used after some selective pre-purification of the sample of interest has been performed. Biological matrices are particularly complex and if not adequately pre-treated, can reduce the efficacy of a chromatographic system and/or altogether destroy the system.

In traditional liquid chromatography, a hollow glass or metal tube is filled ("packed") with a polar slurry (the SP). The mixture to be separated, dissolved in a non-polar MP, is layered on top of the slurry (or, "chromatographic bed") and flows through it by the force of gravity and the pressure of the solvent reservoir above it. [Dimensions of such columns and the nature of the components of their SP are highly variable.] While this type of chromatography is a powerful separation technique, it is also very slow (requiring up to six hours to complete a single "run") and is not readily amenable to automation.

It was discovered that separations could not only be accelerated but also improved if the size of the particles that made up the chromatographic bed was reduced from greater than 100 μm to 5-40 μm and thereby significantly increasing the amount of surface area available for the chromatography. The smaller particle sizes also entailed a five- to ten-fold reduction in the diameter of the columns [typical dimensions: 30 cm x 4.0 mm, L x I.D.] thereby imposing a need for special

pumps to drive the MP through the matrix of the SP (which had now aquired a considerable resistance to flow). A new generation of high pressure pumps was developed to deal with these mechanical challenges. These pumps have gone on to lend their name to a new type of chromatography: high pressure [i.e., normally >1000 psi] (also called "high performance") liquid chromatography, or HPLC.

A variety of materials are available as column packings including silica, alumina and organic polymers, but silica is the most versatile and the most widely utilized. Silica packings are available in three main types of particles: (a) macroparticulate - particles are generally spherical, completely porous and >40 μm in size; (b) microparticulate - particles are spherical or irregularly shaped, completely porous and 3-10 μm in size; (c) pellicular - in this case, glass beads, 20-60 μm size, are coated with a porous layer of silica.

Columns made of the microparticulate silica material offer the greatest flexibility, have the highest chromatographic efficiencies (i.e., attainment of well-defined narrow peaks) and the greatest loading capacities. [The smaller the particle size, the greater the theoretical plate count and the greater the loading capacity.] Furthermore, an analysis can be completed without the need for prior derivatization and within a short period of time (generally less than thirty minutes and often less than ten). This latter characteristic simplifies the development of optimal

separation conditions for a given separation. Finally, microparticulate columns (in HPLC systems) permit swift diagnosis of problems.

These microparticulate beds allow many different types of chromatography to exploit the HPLC systems. For example: the HPLC-version of liquid solid chromatography involves the use of the native silica particles as the SP (this is simple adsorption-chromatography). However, in liquid-liquid HPLC, the particles of the silica have had a liquid chemically bonded to their surface, thereby allowing ion-exchange or partition chromatography.

HPLC systems are of particular interest for biomedical analyses because they permit the simultaneous separation and quantification of trace amounts of physiologically significant compounds. Normally, there are at least six components to the HPLC system: solvent reservoir(s); pump; injector (i.e., a means by which to introduce the sample to be analyzed into the system); column (containing the SP); detector(s) [by which the analyte(s) is(are) quantified]; and data recorder(s) [See Figure 3].

Briefly, the pump draws the MP from the solvent reservoir through the column and the detector. The sample mixture to be analyzed is introduced into the system by the injector which places the analyte(s) into the flowstream of the MP, which, in turn, transfers the analytes to the column. The column separates the components of the mixture into individual bands (as in TLC). Each band eventually migrates out of the column

and through the detector; the detector is, in turn, connected to some recording device which registers a deflection of a pen as each band passes over the active surface of the detector. The tracing resulting from the elution of a single band is known as a peak [See Figure 5 - Generic Chromatogram].

Identification of peaks is on the basis of retention time, as was the situation in TLC. In HPLC, a library of retention times of compounds (standards) which are structurally related to the analytes of interest and present in the sample matrix is established prior to the start of analysis of unknown samples. However, it must be emphasized that coincidence of retention time of a component of the sample mixture with the retention time of a previously injected standard compound is not proof of the identity of the unknown component. Complete characterization of every peak on the chromatogram entails collection of the effluent of the column and subjecting each peak to extensive analysis. This analysis could include GC-MS, determination of relative current ratios (ϕ), enzymatic peak shift and fluorescence emission spectra [Brown et al., 1979]. Obviously, it is impractical to perform such comprehensive analyses on every peak of every chromatogram on an on-going basis. Therefore, these studies are performed on the peaks of interest when the analytical procedure is first being established in the laboratory and at standardized intervals thereafter as a quality-control measure.

One type of bonded-phase HPLC has become notably practicable: reverse-phase. The name stems from the fact that the bonded SP is non-polar while the MP is polar, in contrast to the traditional configuration of these two phases in liquid-liquid chromatography. Analytes are separated on the basis of hydrophobic interaction with the column and elution occurs in decreasing order of polarity of analytes. The most commonly used bonded, reverse-phase packing materials are different alkyl chains, e.g., a C_{18} hydrocarbon chain, linked to the silica particle matrix.

Reverse-phase is best suited to the separation of non-ionic compounds; however, most biologically significant molecules (including the CA and their metabolites) have ionic, or ionizable, functional groups. These render the molecules polar and do not permit retention to take place on a reverse-phase column. [The CA are bases and their metabolites are acids.] It is possible to circumvent this problem by adding a suitable, large, organic counterion to the MP to form an ion-pair with these ionic functional groups [Eksborg et al., 1973 & Eksborg & Schill, 1973]. This is called "paired-ion partition chromatography".

To take full advantage of paired-ion chromatography, the following factors must be manipulated: the nature and concentration of the counterion (for basic compounds, such as the CA, alkyl sulfonates are used [Moyer & Jiang, 1978]); the pH of the mobile phase (low pH encourages ionization of bases and thus aids in the formation of the ion-pair); the polarity

of the the solvent used in the MP (addition of methanol, for example, to an aqueous MP aids the partition process by counteracting the effect of the counterion and thereby "speeding up" the separation); and, to some extent, the nature of the SP backbone. In summary, all of the above factors must be taken into consideration when a column is being chosen for a specific separation [Lim, 1986].

The column used in the assay reported herein was a microparticulate, reverse-phase, bonded-phenyl column (30 cm length). To my knowledge, the use of this column in an HPLC-ECD CA assay is unique. The mobile phase was constructed on the base of a sodium phosphate buffer. Heptanesulfonate was chosen as the ion-pairing agent [Ghanekar & Das Gupta, 1978]; methanol was the organic modifier used to conteract excess retention; and EDTA was added to the mobile phase in an effort to chelate any stray metal ions. The exact basis for the interaction of this column with the CA molecule is not known with certainty. It is presumed that interactions of the planar or π electron cloud of the phenyl ring occur with the ring of the CA, made possible, in part, by the counterion.

Appendix III

Summary of Principles of Electrochemistry

Electrochemistry is concerned with the interconversion of chemical and electrical energies. This includes chemical changes which are produced by electricity (as in the case of electrolysis) and the production of electricity by chemical events.

There are two types of conducting materials - electronic and electrolytic - and some materials display both kinds of conduction. The most prevalent electronic conductors are metals and the most common electrolytic conductors are solutions of acids, bases and salts. The electricity which flows through electronic conductors is due to a stream of electrons; therefore, no net transfer of matter occurs. In the case of electrolytic conductors (i.e., the salt solutions), the carriers of the electricity are ions and, in response to a potential gradient, a transfer of matter takes place.

Normally, electrochemical systems consist of both types of electrical conductors: if electricity is passed through such a system (i.e., it would have to pass through the metal or "working electrode"), a quantifiable chemical reaction occurs at the interface between the two types of conductors. This reaction is referred to as an electrochemical reaction and is further characterized as oxidation or reduction depending on whether the generated flow of electrons is to or

from the electrolytic carrier (i.e., the salt solution), respectively. The flow of electrons so-generated is directly proportional to the concentration of the compound which is the source of these electrons.

The tendency of a compound to be oxidized or reduced in response to an electrical field is referred to as its electroactivity. This tendency can be capitalized upon to create detection systems capable of quantifying analyte(s) of interest for the purposes of chemical analysis. This phenomenon is exploited in the field of voltammetry, one version of which is solid electrode voltammetry.

To be compatible with high pressure liquid chromatography (HPLC), an electrochemical detector (ECD) must be placed in a configuration that is compatible with the conditions of operation of the chromatographic column. Furthermore, to be effective in any particular HPLC system, the ECD, in turn, requires that the mobile phase (MP) be electroconductive (because the MP is effectively one of the components of the electrochemical reaction); the working electrode surface must be chemically inert; and, the analyte(s) to be quantified must be electroactive and ionizable in the chosen MP composition (this latter characteristic is a requirement of the conditions of separation by the column). It is important to appreciate that the working electrode (WE) is also the site of the electrochemical reaction of the analate.

The most common juxtaposition of the critical components of an ECD cell that is encountered in biomedical analyses is

the thin-layer type (See Figure 4) comprising three electrodes connected to a stable power supply and an electronic control board. The column effluent (MP plus or minus analyte) is directed to a small, flat compartment (typical volume: 10 μL) within a sandwich of Kel-F plastic. One side of this compartment has an embedded planar, working electrode which is held at a fixed potential (e.g., +0.6 V) by the power supply [Kissinger, 1973]. The working electrode is usually made of carbon (either as carbon paste or glassy carbon). As the analyte passes over the WE surface, it is oxidized (or reduced) in response to the electrical field and generates a flow of electrons (current) which is quantified by the electronics.

However, the resistance of the MP (i.e., the electrolytic conductor) changes constantly as it passes through the HPLC system and over the WE; this, in turn, affects the potential of the working electrode and affects its ability to oxidize or reduce an analate. To compensate for this, an auxiliary electrode is used and is held constant relative to a reference electrode (usually Ag/AgCl). Any variation between the known value of the reference electrode and the measured value of the auxiliary electrode is due to the iR drop in the compartment (where "i" is the current passing through "R", the uncompensated resistance) caused by the MP [BAS, LC-4B Manual, 1982]. This discrepancy can be compensated for electronically and eliminated. This allows the WE to maintain a constant potential regardless of variations in the

resistance of the solution (i.e., the electrolytic conductor is not constant) and ensures that any additional current that is generated in the cell will come largely, if not exclusively, from the analyte of interest.

The electrical current (measured in nanoamperes) resulting from the oxidation (or reduction) of the analyte is converted to a voltage and amplified by the electronics of the detector. This voltage is then quantified by a data-recording device and reported as a peak height/area and/or analyte concentration. The current produced in the cell is directly proportional to the concentration (that is, the number of molecules flowing by the WE) of the analyte(s) in the solution. The range switch on the operating controls of the ECD determines the sensitivity of the output of the WE: graded selections are available from 0.1 to 500 nA Full Scale Deflection (F.S.D.). A setting of 5 nA means that a 2.5 nA output signal would correspond to a recorder pen deflection of one-half full-scale.

The signal generated by the MP (and controlled by the auxiliary and reference electrodes) is the source of the "baseline output" that is referred to in discussions of the output of the HPLC-ECD system. It is important to appreciate that any current produced by the analyte as it, in turn, passes over the WE surface will be superimposed on this tonic signal. [To be remembered: The MP is a vital part of the electrochemical reaction and is constantly flowing through the electrochemical compartment in a wet system, i.e., liquid

chromatography.] Obviously, the ability to discriminate the current produced by an analyte will depend on the quantity of background signal generated by the MP; the less background signal present, the more visible will be the current from the analyte. In fact, the stability, linearity, reproducibility and utility of an electrochemical detector at the nanoampere level depends entirely on the control of this background signal.

The characteristic electroactivity of a compound offers a certain basis for discrimination but this is insufficient to ferret out individual components of a complex mixture. This latter issue is the function performed by chromatography. The role of the ECD, then, is to quantify individual compounds once they have been isolated by the chromatography.

There are, in fact, two types of electrochemical detectors: the coulometric and the amperometric. The coulometric detector permanently oxidizes (or reduces) 100% of the molecules passing over the WE and therefore generates a much larger current than the amperometric detector, which only oxidizes about 10% of the molecules. In practice, the higher yield of the coulometric detector does not seem to improve its sensitivity because the background noise level is also significantly increased. This results in a poor signal:noise ratio, thereby nullifying any benefits from the larger signal. Alternatively, the amperometric detectors permit collection of the analyte following passage through the cell and thus further purification is possible, if necessary. Therefore,

the amperometric detector is most often encountered in biomedical applications (and has become the prefered detector in the field of CA analysis).

APPENDIX IV

Information and Consent Form

Catecholamines in Subarachnoid Hemorrhage

PATIENT: _____ DATE: _____

The patient named above has had some bleeding inside the head from the rupture of a cerebral aneurysm. Repeat bleeding will be prevented by an operation to clip the aneurysm but another potentially serious problem called vasospasm may occur to complicate the management. Vasospasm is usually delayed for at least several days after the initial rupture of the aneurysm and results in a narrowing of blood vessels to the brain. This narrowing may impair blood flow to the brain resulting in temporary or permanent damage to brain tissue comparable to the effects of a stroke. Vasospasm is fairly common, and often fatal, but does not <u>always</u> occur after rupture of a cerebral aneurysm. The reason(s) for its appearance in certain patients but not in others remain(s) a mystery and therefore, an effective treatment for this condition has not yet been found.

A study is going on at this hospital to investigate at least one potential cause of vasospasm: excess sympathetic nervous system activity. The sympathetic nervous system is responsible, in part, for secreting powerful hormones, namely adrenaline and noradrenaline, to help our bodies function in both normal everyday life and under conditions of great

stress. This latter function is in fact the reason that adrenaline and noradrenaline have been nick-named the "stress hormones". It seems that sometimes too much hormone is secreted and this can be potentially dangerous. For example, blood is not normally present in the spaces surrounding the brain and blood contains many substances which cerebral arteries find irritating and they respond to this irritation by vasoconstriction or narrowing. This results in the phenomenon of vasospasm. The hormones adrenaline and noradrenaline are normally present in the blood in very tiny amounts but under the stress of a ruptured aneurysm and admission to hospital, etc., the concentration of these hormones in the blood rises considerably and stays elevated. We are trying to find out if this in fact worsens the progress of this group of patients.

Our study involves the taking of periodic blood samples (two times a day) from the time of admission throughout the stay in hospital. The blood is collected by an experienced individual under aseptic conditions. The blood is then analyzed in a laboratory to see how much, exactly, of the hormones it contains. The volume of blood taken is small (about two teaspoons) and it is done through the intravenous or intra-arterial catheter that is already in place, so no new changes will be added to the care of the patient, nor will the patients well-being be affected by this minimal sampling of blood. In fact, there will be <u>no</u> change made whatsoever to the treatment regimen of the patient; this study will only

follow the patient throughout his/her clinical progress. The results will not be released until the study is completed to ensure unbiased interpretation of the results. The procedures will not provide any specific benefit to any one patient at this stage but any additions to our knowledge and understanding of the underlying mechanisms of vasospasm would allow us in future to devise an effective treatment, if not prevention, for this condition. You are invitied to pose any questions that you might have regarding any aspect of the procedures to be followed. It is understood that refusal to take part in this study or the decision to withdraw from it will not in any way affect the patient's present or future care.

I _____, having read the above and understood the implications of the procedures described hereby undertake to participate in this study.

SIGNED: _____ WITNESS: _____

If the patient is unable to sign or is under 18 years of age, complete the following:

RELATIONSHIP TO PATIENT: _____

SIGNED: _____ WITNESS: _____